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"COPPER UPTAKE AND TOXICITY IN FREE LIVING AND IMMOBILIZED CELLS OF *Nostoc calcicola* AND IN COPPER TOLERANT STRAIN"

THESIS SUBMITTED TO

BUNDELKHAND UNIVERSITY, JHANSI

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

By

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DECLARATION

I, **Omendra Kumar Prajapati**, hereby declare that the thesis entitled "**Copper uptake and toxicity in free-living and immobilized cells of *Nostoc calcicola* and in copper tolerant strain**" is my own work conducted under the supervision of Dr. Pramod Kumar Pandey. Reader, at Department of Biotechnology, J.C. Bose Institute of Life science, Bundelkhand University, Jhansi as approved by Research Degree Committee. I have put in more than 200 days of attendance with the supervisor at the centre.

I further declare that to the best of my knowledge the thesis does not contain any work, which has been submitted for the award of any degree either in this university or in any other University / Deemed University without proper citation.

Omendra

(Omendra Kumar Prajapati)

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(Omendra Kumar Prajapati)

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Dr. Pramod Kumar Pandey
Reader,
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CERTIFICATE

I have great pleasure in forwarding the thesis entitled "**Copper uptake and toxicity in free-living and immobilized cells of *Nostoc calcicola* and in copper tolerant strain**" submitted by **Mr. Omendra Kumar Prajapati,** for the Degree of Doctor of Philosophy in Biotechnology at this University, Mr. Prajapati has completed the prescribed term of Research work under my supervision. The candidate has put-in an attendance of more than 200 days with us. To the best of our knowledge and belief the thesis:-

(1) embodies the work of the candidate himself;

(2) has been duly completed;

(3) fulfills the requirements of the ordinance relating to the Ph. D. degree of the university; and

(4) is up to the standard both in respect of contents and language for being referred to the examiner.

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This is to certify that **Mr. Omendra Kumar Prajapati** S./o. Mr. N. L. Prajapati has completed his research work entitled "Copper uptake and toxicity in free-living and immobilized cells of *Nostoc calcicola* and in copper tolerant strain", for the Degree of **Doctor of Philosophy in Biotechnology** from Biotechnology Research Laboratory, Department of Biotechnology, Bundelkhand University, Jhansi, under the guidance of Dr. Pramod Kumar Pandey. He has carried out his Ph.D. (Biotechnology) Research work (Registered on 20th October, 2005; Registration No:- B.U./ Administration / Research / 2005 / 3100-02) sincerely with managerial capacity. This Research work is original and has not been submitted to any other University for Degree or Diploma.

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ABBREVIATIONS

AA-N :	Allen and Arnon's growth medium without nitrate
ANSA :	1-amino -2- nepthol-4-sulphonic acid
ATP :	Adenosine triphosphate
ADP :	Adenosine diphosphate
CPM :	Count per minute
Cu :	Copper
Cu^s :	Copper sensitive strain
Cu^r :	Copper resistant strain
Ca^{+2} :	Calcium ions
Chl *a* :	Chlorophyll *a*
D :	Dark
DCCD :	N,N'-dicyclohexylcarbodimide
DTT :	Dinitrophenol
EDTA :	Ethylene diamine tetra acetic acid
Fe-EDTA :	Iron-containing ethylene diamine tetra acetic acid
GS :	*Glutamine synthetase*
h :	Hour
HEPES :	4-(2-hydroxy ethyl)-1-piperazine ethane
Kb :	Kilo base
Kda :	Kilo Dalton
L :	Light
µg :	Microgram
µl :	Microlitre
µM :	Micromolar
ml :	Millilitre

min :	Minutes
Mg^{+2} :	Magnesium ions
N_2 grown :	Culture raised without combined nitrogen source
NAD :	Nicotinamide adenine dinucleotide
NADPH :	Reduced nicotinamide adenine dinucleotide phosphate
N R :	*Nitrate reductase*
pCMB:	p-chloromercuribenzoate
PO_4^{3-} :	Phosphate ions
PS :	Photosystem
p-NPP :	p- Nitrophenol phosphate
p-NP :	p- Nitrophenol
Pi :	Inorganic Phosphate
S.E. :	Standard error for three independent
Tris :	2- amino-2-(hydroxyl-methyl) propane-1,3-diol
UV :	Ultra-Violet
V :	Volt
Vol :	Volume
v/v :	Volume / Volume
w / v :	Weight / Volume

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Looking back and remembering the past is never easy, more so when we tend to remember trifles of things that have contributed to our present. But I am taking this opportunity to articulate my feelings for all those people who matters most in my life. It is by the grace and will of the Almighty that I have reached the finishing tape with a thesis in my pocket and it feels good! Sometimes I have been running against the wind, but most often I have had the wind behind me. I would like to take this opportunity to humbly express my deepest gratitude for the innumerable gesture of help, cooperation and inspiration that I have received from my teachers, elders, and friends and well wishers during the course of this work.

This Research work, like all worthwhile pursuits has been a team effort. I wish to express my sincere gratitude to all those involved. At first, I want to thank the Vice-Chancellor of Bundelkhasnd University, Jhansi for allowing me to pursue my Ph.D. in Biotechnology from the University.

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completion of the Ph.D. degree course, giving the words of strength, appreciation and helping me in every step in my University campus.

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God cannot be with everyone all the times. That's why he made parents. A child can never thank his parents but he owes his existence to them. I will also not show my courage to thank my parents, but I would just pray to God that never in my life should I do anything that can hurt them. My honest, simple and immensely will-powered parents never wanted anything from me. I can never be like you Papa and Mom, but just give me your blessing so that I can never be disloyal to your limitless trust, love warmth and care. Let me not make your sacrifices go to waste.

My sisters Garima and Geeta have always been a source of stimulation for me. Their immense faith in my capabilities always inspired me to work harder. Their innocent love; care and affection always gave me a flesh least of life.

Last but not least, I am thankful to the God, for putting such wonderful people in my life and bestowing his Warmth and Affection through them.

.....Omendra Kumar Prajapati

DEDICATED

TO MY

LOVING PARENTS

PREFACE

The subject of environment pollution has attracted the common public and politicians as well. The third world countries including India, through much anxious to industrialize fast are, however, little aware of the environmental consequences with regard to the nature and geographical distribution of wastes or the impact of extreme climatic regimes. The technological warfare has also resulted in the increased output of pollutants and air, water and soil atmosphere, all have competence to retain such pollutants or circulate them amidst each other to ultimately thereafter the very survival of living organisms unless some alternative mechanisms are adopted by a living cell to overcome the pollution load.

Copper is a well-known plant micronutrient, an algaecide as well as known fungicide, metal component of plastocyanin, and the enzyme superoxidase dimutase. Copper like other heavy metal, is also growth toxic to algae including cyanobacteria at fairly low levels in the range of micro-molars. The present Research work, therefore reflects the compilation of investigations on the physiological and biochemical and physiological aspects of cyanobacterial metabolism with emphasis on copper uptake, and phosphorus and nitrogen metabolism in response to intracellular Copper, ^{14}C-incorporation, ATPase activity and isolation of Cu tolerant / resistant (Cu^r) strain

from the Cu sensitive (Cu^s) Nostoc calcicola. As mode of development of metal tolerance / resistance remains little explored in such prokaryotes compared to higher plants.

The resistant traits of Nostoc calcicola Breb were of (a) exclusion type, (b) extrusion type, (c) accommodation type. While stability was tested by successive transfers to Cu-less medium, metal extrusion studies were aimed at knowing the possible correlation between Cu efflux and influx in terms of minutes and hours i.e., short term experiments.

The optimal utilization of liquid scintillation counter, spectrophotometer and Gas chromatograph has enabled me to cover the vital aspects of metal analysis and ^{14}C-incorporation, without which I could have been left with the only option to get satisfied with growth measurement or at the most, protein estimation to characterize Cu toxicity.

There may be to be short comings either in experimental aspects or in their interpretations of this research study. Suggestions for further improvement or otherwise also from those who will be going through this piece of work are welcome.

CONTENTS

CHAPTER – 1.

INTRODUCTION

CHAPTER – 1.
INTRODUCTION

Environmental pollution and contamination have become a key focus of concern. Changes in technology and manufacturing practices are providing relief to these problems. However, some of the present methods of environmental cleanup result in the production of harmful by-products. Environmentally friendly process needs to be developed to clean up the environment without creating harmful waste products.

Any change, which has the potential to adversely influence to biological and non-biological equilibrium of environment is called pollution. Pollutant can be substance just like dust, smoke and chemical metal such as Al, As, Cu, Cr, Cd and Pb etc. or a factor that on release into the environment has an actual or potential to adverse effect on natural environment. Among pollutant, metal significantly contribute in environmental contamination.

Over the last thee-decades, environmental problems are multiplying and becoming complex due to the increasing population, uncontrolled urbanization and industrialization. Disposal of untreated or partially treated industrial wastes into surface water is one of the major environmental problems in developing countries. The discharge of fly-ash and untreated industrial effluents or semi treated hospital and sewage waste into water bodies is causing adverse effect on aquatic micro-flora and macro-flora thus disrupting the natural balance either in

the form of killing of sensitive species or dominance of a particular species which is capable to tolerate the high concentration of metals present in aquatic system. But as we aware the algae along with the other aquatic plants are the primary source of energy in the fresh and marine water ecosystem (Dwivedi *et al.*, 2004). Thus they have to play vital role for cleaning the contaminated sites and maintained the aquatic food chain without any hazardous contents.

Due to rapid growth of industrialization and human civilization has resulted in massive mobilization of heavy metal. Elevated levels of heavy metal in the water can be attributed both natural and anthropogenic sources. The most significant source of metal is weathering of rocks and volcanic activities from which the released metal finds their way into the water bodies. A large quantity of metal also suspended in the atmosphere from where they can reach the water though dry deposition and with the precipitation. The pollution sources of heavy metals increase their concentration of metals in a localized area reaches some time to significantly higher levels and become deleterious to biological populations and human beings. The anthropogenic sources of heavy metals include coal or burning power generation plants, metal processing industries, electric supply line, incineration waste disposal units, building materials auto emission smelting, mining etc.

One of the recent trends is to search for new photosynthetic organism with high growth rates; high biomass yield and high utilization potential, which could be mass cultured in waste water and play a duel role of cleaning the water and also serving as a source of food, fertilizer or fuel. Cyanobacteria, the oxygen evolving

photosynthetic prokaryotes are found in variety of aquatic and terrestrial habitats in nature as well as in association with the organism. They appear to be ideal organisms, since them, show high flexibility to adapt themselves to varied environment and are relativity inexpensive for mass cultivation in view of their trophic independence to carbon and nitrogen. Cyanobacteria are phylogenetically the oldest group of oxygen evolving photosynthetic prokaryotes, occupying an important place in both aquatic and terrestrial ecosystem because of their ability to utilize atmospheric nitrogen (Venkataraman *et al.*,1982). Cyanobacteria interact with pollutants in a variety of ways namely:-

(1) Metabolize or use the pollutants as nutrients,
(2) Detoxify or degrade them,
(3) Accumulate or absorb to the cell.

The algae accumulate heavy metals; this capacity can also be used for purification of metal enrichment and recycling of valuable metals (Kessler, 1986). Such an areas merits attention as many aspects of metal-algal interactions still remain unexploited in biotechnology. Copper is a micronutrient, required in a micro concentration. Since present thesis focuses around cyanobacteria among the algae as target cells and Cu uptake studies, emphasis is mainly around the cellular intake of such metals and the fate of selected physiological and biochemical reactions. Cu, the well-known micronutrient for growth and maintenance of several algae, is also an essential component of Plastocyanin (Levine, 1969; Plesincar and Bendall, 1970). 3μM Cu is optimum concentration for cellular growth for *Nostoc calcicola* but as we gradually increase concentration, it becomes toxic. 5μM is lethal concentration of copper. (Prajapati and Pandey, 2007). This is due to

inhibition of cellular and metabolic reactions. Inhibition of chloroplast reactions by copper; has been mainly though the inhibition of electron transport at the side of P.S.-II (Cedano-Molandonado *et al.,* 1978), direct inactivation of ferredoxin (Shioi *et al.,* 1978), O_2-dependent oxidation of –SH groups on a coupling factor and inhibition of ATP synthesis (Uribe and Stark, 1982).

Copper is taken in by cyanobacterial cells involving a rapid initial step (adsorption), followed in sequence by metabolism dependent intracellular cation intake. Since photoautotroph derive most of their ATP though photosynthesis and metal import dependent on the membrane potential, non-photosynthetic conditions on the presence of metabolic inhibitors or uncouplers hampers the process indirectly. Metal adsorption on the cell wall is the primary site of metal attack as a consequence of S-metal-S bridge formation (Passow and Rothtein, 1960; Passow *et al.,* 1961; Kamp-Nielsen, 1971), and the thiol-disulphide interchange has been achieved in many metal toxicity / uptake experiments (Agrawal and Kumar, 1977; Singh and Pandey, 1981; Singh and Yadava, 1985; Rai and Raizada, 1985, 1987; Verma and Singh, 1990). It is quite satisfying to perform metal toxicity / uptake experiments in buffered media; nevertheless, it does no account for the co-existence of essential ions in nutrient media or a multi-metallic combination (essential and non-essential ones) in natural environment. Metal interactions range from antagonism to synergism and the nature and extant depends on relative concentrations and sequence of additions (simultaneous or subsequent). Antagonism arises though a competition among cations on the cell surface results in synergism; one metal might enhance cell permeability to the other. Metallic combination used in numerous investigators indicate that Zn

or Ca represent a major group of antagonist and the latter principal non-heavy metal factor regulating Zn and Cd bioaccumulation by freshwater algae.

The metal-induced inhibitions of various metabolic events described before, point towards the blockage of cell energy supply / synthesis and reductants as such cations attack the cell membrane. The ultra-structural details suggest that heavy metals find their home in phosphate bodies in cyanobacterial cells with the exception of a few being fixed in the cell wall itself (Jensen *et al.,* 1982). This temporary relief, however, goes risky following depolymerization of the phosphate reserve during starvations caused by the metal itself or any other stress.

An appreciable bulk of information is available on the fate of algal metabolism under heavy metal-stress and in majority of events, the non-essential and even the essential metal cations (beyond a certain threshold limit) inhibited the general growth as "long-term" and vital physiological processes as "short-term" effects (Whitton, 1970; Davies, 1978, 1983; Sorentino, 1979; Huntsman and Sunda, 1980; Rai *et al.,* 1981; Stokes, 1983; Drbal *et al.,* 1985). All such investigations converged in recognizing algae as monitors of heavy metal pollution (Whitton, 1984) or as ecological indicators (Shubert, 1984). However, there is a point of appreciation as fairly low concentration of Ni or Pb (Henriksson and DaSilva, 1978) and Hg (Stratton *et al.,* 1979) also stimulate nitrogen fixation in cyanobacteria. The question arises whether the algal cell encounters heavy metal cations only at the cell exterior or allows their active / passive intra-cellular transport and the subsequent compartmentalization. The present endeavor is, therefore,

an attempt in comparing Cu uptake by free and immobilized cells of the diazotrophic cyanobacterium *Nostoc calcicola* Breb, and the inhibition of a selected group of physiological and biochemical reactions. Cell immobilization in cyanobacteria has resulted in the functional longevity and higher total yields of hydrogen evolution, improved *nitrogenase* activity and ammonia production (Vincenzini *et al.,* 1981; Brouers and Hall, 1985, 1986; Hall *et al.,* 1985) and also metal uptake (Singh *et al.,* 1989).There has been similar report on the undiminished capacity for photosynthetic O_2 evolution and glycolate excretion (Day and Godd, 1985) and on the rapid removal of Hg from the growth medium in *Chlorella emersonii* (Wilkinson *et al.,*1989).The recent reviews have emphasized on the feasibility of the immobilized system, covering a variety of organisms (Fukui and Tanaka,1982; Papageorgiou, 1987; Brouers *et al.,*1989).

The cyanobacterial cells almost analogous to other algae or bacteria, take up heavy metals involving a faster initial reaction (adsorption), followed in sequence by the slower, metabolism-dependent intracellular cation intake. Such events seem to be a rule in case of Zn (Shehata and Whitton, 1982), Cd, Cu and Zn (Les and Walker, 1984), Cd (Singh and Yadava, 1985), Cu (Singh,1985), Al (Pettersson *et al.,*1986) and Ni (Campbell and Smith, 1986). All such studies collectively argue that metal transport depended on the membrane potential of the target cell and that invariably decreased under non photosynthetic conditions or in response to metabolic inhibitors / uncouplers of electron transport. The ultimate sink for such cations brought to light though morphometric studies or to be more precise, though X-ray energy dispersive analyses allowing a multi-elemental coverage, has been the polyphosphate bodies although the

relatively immobile elements also found their home in the cell wall (Jensen *et al.,* 1982). The major reshuffle at the cell interior includes increased thylakoidal surface area and excessive intracellular membrane whorls (Rachlin *et al.,* 1982), increased cell lipid material, mobilization of protein reserve and the elevated volume of polyphosphate bodies (Rachlin *et al.,* 1985). Possibly, these are the defense mechanisms devised by cyanobacteria in addition to *metallothionein*, the metal binding protein reported earlier (Olafson *et al.,* 1979; Ravendra *et al.,* 2002; Ren *et al.,* 1998).

The apparent variation in metal uptake by cyanobacteria or other microbes has been taken as the species specific problem that might include events like metal resistance / tolerance mechanisms like precipitation, volatilization and hydrolysis as well as the specific efflux means adopted by the cell. A characterization of Ni-resistant mutants of *Synechococcus* ATCC 1746 revealed that while a class of mutants synthesized a large amounts of intra-cellular polymer that complexes Ni with almost a 300-fold difference over the internal cytoplasm concentrations; the mutants in the other could complex Ni extracellularly by synthesizing low molecular weight polar ligands (Wang and Wood, 1984). It is in this context that the Zn-tolerant strain of *Anacystis nidulans* permitting metal intake comparable to that of the wild-type (Shehata and Whitton, 1982) would belong to the former group while the Cd-tolerant strain of *Anacystis nidulans* allowing metal intake (Singh and Yadava, 1986).

Laboratory experiments dealing with population density *VS.* heavy metal uptake have revealed that dense cultures were less sensitive to cations that were otherwise highly toxic to the same

organism at the reduced population size (Delcourt and Mestre, 1978; Wikfors and Ukeles, 1982); the possible reason being the magnified intercellular distance at low cell titer (Itoh *et al.,* 1975) contrary to reduced cell surface area in dense cultures (Kurek *et al.,* 1982). Similar correlation has also been demonstrated in *Chlorella* (Gipps and Coller, 1980), *Anacystis* (Singh and Yadava, 1985) and *Nostoc* (Schecher and Driscoll, 1985).

The observed lowered metal uptake at alkalinity may put the alkalophilic cyanobacteria in the advantageous position over others, however, a reverse situation has also been encountered in some algae and even cyanobacteria (Steeman-Nielsen and Kamp-Nielsen, 1970; Gachter, 1976; Hargreaves and Whitton, 1976; Yanagimoto *et al.,* 1983; Les and Walker, 1984; Schecher and Driscoll, 1985; Singh and Yadava, 1985; Rai *et al.,* 1990). There are extensive reviews covering the role(s) of humic and fulvic acids in the chelation of metal ions (Schnitzer and Khan, 1978; Boggs *et al.,*1985), and based on the relative binding and stability constants, Kerndorff and Schnitzer (1980) grouped such metals as (a) strong binders:- Fe^{3+}, Hg^{2+}; (b) intermediate binders:- Cu^{2+}, Pb^{2+}, Al^{3+} and (c) weak binders:-Ni^{2+}, Cr^{3+}, Zn^{2+}, Mn^{2+}, Co^{2+} and Cd^{2+}. Apart from the synthetic complexans or natural ones just described, several algae also produce strong Cu-complexing agents that reduce the free metal ion concentration (Van den Berg *et al.,* 1979; McKnight and Morel, 1979; Laegried *et al.,* 1983; Vasseur *et al.,* 1988). Hodson *et al.* (1979) pointed out that chelation is the single and most vital abiotic factor in the amelioration of Cu-toxicity in aquatic ecosystems, therefore, the motive behind using pond water, soil extract or the spent medium, has been to

compare their relative metal complexing efficiency in terms of reduced Cu uptake in free and immobilized *Nostoc calcicola* cells.

Exposure to heavy metals may alter the transport of nutrients and the resultant intracellular nutrient levels minimize the fidelity of nucleic acid, protein synthesis that eventually leads to viability loss. Such alterations in nutrient transport are the consequence of competitive or non-competitive interactions between heavy metal cations and the macro / micro-nutrients for cellular uptake (Vallee and Ulmer, 1972). While, competitive inhibition in such cases involves a direct encounter between ions prior to uptake, the non-competitive type accounts for the short supply of energy and reductants. The utilization of any form of inorganic nitrogen though photoautotrophic route in cyanobacteria has to depend on the availability of ATP and reductant and also, the ammonium taken up from the outer medium or internally generated by either nitrate reduction or dinitrogen fixation, is assimilated though its combination with carbon skeleton *via* the energy-dependent GS-GOGAT pathway (Guerrero and Lara, 1987). There are numerous reports that heavy metals also inhibit *nitrogenase* activity (Henriksson and Da Silva, 1978; Stratton and Corke, 1979; Singh and Pandey, 1981; Pettersson *et al.,* 1985; Singh *et al.*1987), *glutamine synthetase* (Ip *et al.,* 1983; Singh *et al.,* 1987) and phosphorus-phosphate uptake (Singh and Yadava, 1984; Pettersson *et al.,* 1988). As the nature and extent of inhibition in such cases, differed from metal to metal, it is hard to make a generalization.

As the metal inhibition of various metabolic processes ultimately leads to nutrient starvation or even the ultimate cell death, it is worth trying the metal intake / transport in organisms already facing

nutrient deficiency. Since the present effort is confined to carbon, phosphorus, nitrogen and sulphur, a comparison has been made to point out the possible consequences of cell starvation / recovery with reference to Cu uptake by free as well as immobilized cells. As the terms photosynthesis and CO_2-fixation are interchangeable, the reduced ferredoxin and ATP generated in photosynthetic reactions, are also consumed in assimilatory processes other than CO_2-fixation, i.e., the assimilation of inorganic nitrogen in light. This perhaps explains the non-availability of carbon skeleton in photosynthetic organisms exposed to dark. Nitrogen deficiency leads to loss of phycocyanin and increase in glycogen content (Alien and Smith, 1969) and utilization of polyhedral bodies as nitrogen source (de Vasconcelos and Fay, 1974).The carbohydrate reserves accumulated in N-starved cells can apparently substitute for fresh products of CO_2-fixation. Sulphur starvation inhibits protein synthesis but not the production of Arg-poly (ASP) or accumulation of polyphosphate bodies (Lawry and Simon, 1982). The quality of polyphosphate to form insoluble salts with various heavy metal ions provides a potential tool for scavenging such ions from the cell cytosol. Ultra-structural investigations in such direction indicate that salts of polyphosphate bodies really *in situ* (Jensen *et al.,* 1982). However, it is yet to be ascertained if such polymers could be utilized as a tool for bio-concentration of desired elements because phosphorus-deficient conditions lead to their depolymerization or the eventual release of bound metal(s).

The present Ph.D. (Biotechnology) thesis has been divided into 9 main chapters following a brief Introduction (Chapter I), Review of the literature (Chapter II), Objectives (Chapter III), Materials and Methodology have been dealt with in Chapter IV, the two chapters

that follow (V and VI), embody Results and Discussion, each incorporating seven main sections.Section-1 describes the Cu-uptake in free and immobilized cells of *Nostoc calcicola* and also compares the extent of active and passive Cu uptake in both the states of cells. Section-2 contains the factors regulating Cu-uptake (light generated energy and inhibitors / uncouplers). Section-3 deals with interaction of Cu with phosphate uptake along with Ca^{2+}-dependent and Mg^{2+}-dependent *ATPases in vivo* and *in vitro.* Section-5 is contributed by observations on the activity of *nitrogenase* and *glutamine synthetase* under Cu-stress. Photosynthetic aspects dealing with CO_2 assimilation are narrated in Section-6. Section-7 describes the isolation and characterization of copper tolerant strain of *Nostoc calcicola.* Summary and Conclusion are dealt with Chapters VII and VIII. Chapter IX contains References.

CHAPTER – 2.

REVIEW OF THE LITERATURE

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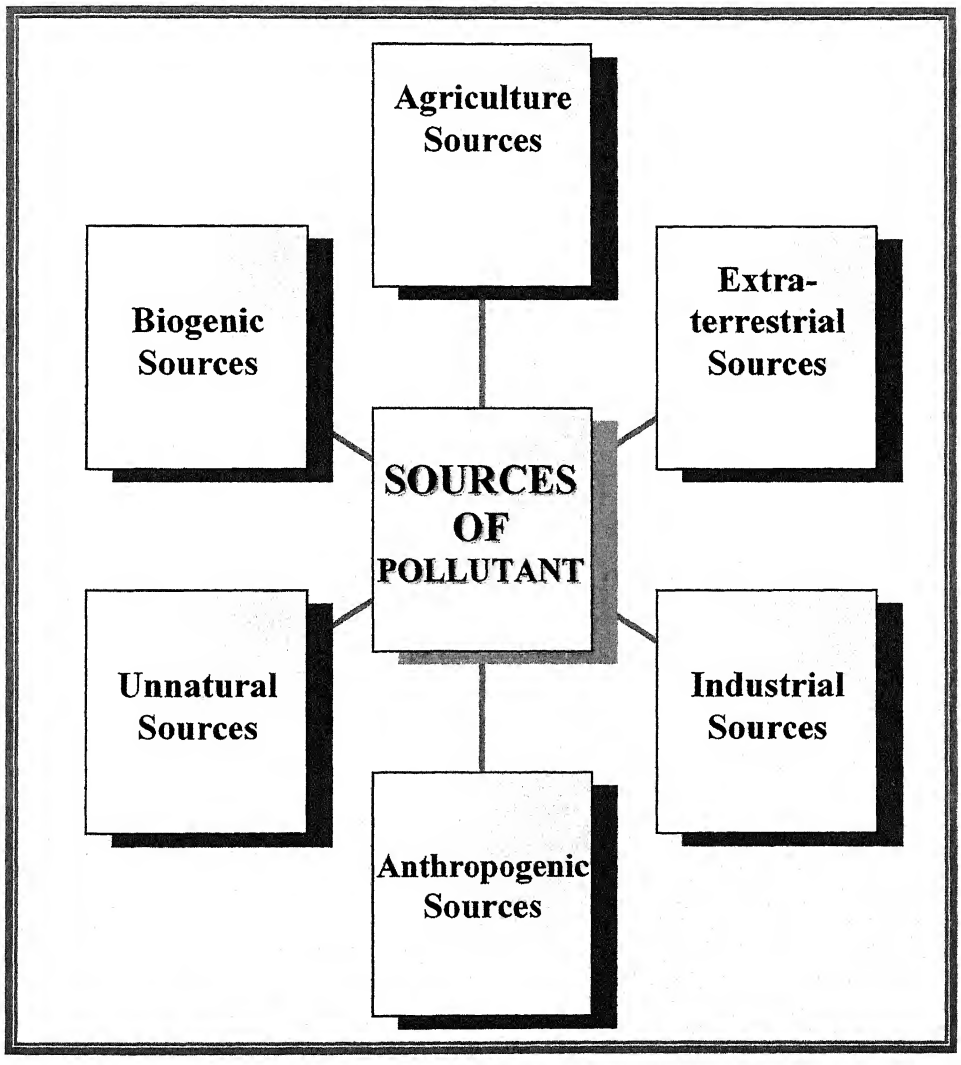
REVIEW OF THE LITERATURE

Pollution of the biosphere with toxic metals has accelerated dramatically since the beginning of the industrial revolution. The primary sources of pollution are the burning of fossil-fuels, mining, smelting of the metalliferous ores, municipal waste, fertilizers, pesticides, and sewage (Nriagu *et al.,* 1996). Toxic metal contamination of soil, aqueous waste steams, ground waste pose major environmental and human health problems which still need an affordable and economic solution.

Industrial development has created an important number and volumes of wastewater coming from the manufacturing processes; containing pesticide, heavy metal and other toxicant (Travieso *et al.,* 1998). Increased circulation of toxic metals though water and their inevitable transfer of a higher tropic food chain, are likely to cause important environmental hazardous (Mallick and Rai, 1990). Activities associated with modern society, industry, power generation, transportation has subjected the environment to release of many toxic chemicals interact with atmospheric component causing pollution.

The progress emerging from the technological advance in terms of output of pollutants in general and the heavy metals in particular have virtually heightened social and political awareness world-over. Heavy metals to phytotoxic concentrations generally prevail in soils

Figure: A Diagrammatic representation of different sources of pollutant



under natural conditions though weathering but the major contributions are shared by industrial and agricultural activities. Akin to other living organism, plants down to algae, are susceptible to heavy metal load, and the only way out is to: (1) alter their metabolism, (2) accommodate metal ions and (3) develop tolerance / resistance.

2.1. THE CYANOBACTERIA:-

Cyanobacteria are gram negative eubacteria (Stanier and Cohen-Bazire, 1977) containing plant like oxygenic photosynthetic apparatus remarkably similar in functional, structural and molecular aspect to that of eukaryotic chloroplast (Lang, 1968; Allen, 1968). They occupy an important place in both aquatic as well as terrestrial ecosystems (Stewart, 1980). Cyanobacteria are now considered the largest, most diverse and most widely distributed group of photosynthetic prokaryotes (Singh, 1950; Desikachary, 1959; Stanier and Cohen-Bazire, 1977; Rippka *et al.*, 1979; Carr and Whitton, 1982; Sprent and Sprent 1990; Singh *et al.*, 1995; Adams, 2000; Rai *et al.*, 2000; Whitton and Potts, 2000).

These organisms are morphologically, physiologically and developmentally diverse and form one of the major eubacteria phyla (Woese and Fox, 1972; Woese, 1987; Sivonen *et al.*, 1997; Whitton and Potts, 2000).

Cyanobacteria possess the ability to synthesize chlorophyll *a*. Typically; water is the electron donor during photosynthesis, leading to the evolution of oxygen. Cyanobacteria, recently have also been characterized by their ability to form the phycobilin and phycocyanin

pigments. High concentration of these pigments under some condition leads to the bluish color of the organism and hence the organisms are commonly known as cyanobacteria or blue green algae. However, it has increasingly become clear that at least some oxygen evolving prokaryotes such as *Prochlorothrix,* which do not possess phycobilins, but do form chlorophyll *b* in addition to chlorophyll *a* and quite closely related to organism with phycocyanin.

Cyanobacteria have long history of adaptation to earth's environments since the period of Pre-Cambrian era. They are now the largest group of photosynthetic prokaryotes, as judged by their wide spread occurrence, frequent abundance and morphological diversity (Rippka *et al.*, 1979; Whitton, 1992; Schoof, 2000), symbiotic interactions with free living diazotrophic cyanobacteria and other organisms and plants ranging from algae to angiosperm (Stewart *et al.*, 1983; Allnutt, 1996).

Cyanobacteria are probably one of the most important groups of organisms of serious global ecological importance and respond to the change in their environment (Nichols, 2000; Rai, 1997; Bhaya *et al.*, 2000) among the behavioral response of aquatic cyanobacteria. Cyanobacteria are also important in many terrestrial environments. They often play a key role in maintaining the stability of the surface crust of semi desert and the fertility of soils used for farming in and regions (Whitton, 2000).

2.1.1. NUTRITIONAL PROPERTIES OF CYANOBACTERIA:-

The common nutritrional properties of cyanobacteria have been recognized for many decades (Stanier and Cohen-Bazire, 1977; Carr and Whitton, 1982; Gallon, 1980; Doolittle, 1979; Fogg, 1988; Rippka *et al.*, 1979; Shilo *et al.,* 1982; Singh, 1961; Desikachary, 1959; Venkataraman, 1961). All members of this group are aerobic photoautotrophs; the reactions of oxygenic photosynthesis are used to drive biosynthesis at the expense of inorganic nutrients. In the dark, cyanobacteria display an endogenous respiratory metabolism that is subject to immediate and severe inhibition in the light (Pelroy and Bassham, 1973; Stanier and Cohen-Bazire, 1977; Carr and Whitton, 1982; Matthijs and Lubberding, 1988; Bottomley and Van Baalen, 1978; Raboy *et al.*, 1976; Khoja and Whitton, 1971; Sprent and Sprent, 1990). The phenomenon of obligate photoautotrophy is wide spread among cyanobacteria (Fogg, 1982, Stanier and Cohen-Bazire, 1977; Carr and Whitton, 1982; Doolittle, 1979; Rippka and Stanier, 1978). However, photochemoheterotrophy and photoheterotrophy has been observed in few strains of cyanobacteria (Bisen, 1984; Cossar *et al.*, 1984).

Four classes of inclusions appear to be present in the vegetative cells of cyanobacteria: polyphosphate granules; glycogen granules; cyanophycin granules and carboxysomes. Other inclusions present in the vegetative cells of some cyanobacteria are poly-β-hydroxybutyrate granules and gas vesicles (Stanier and Cohen-Bazire, 1977; Smith, 1982; Healey, 1982; Carr and Whitton, 1982; Doolittle, 1979;

Venkataraman, 1961; Desikachary, 1959; Singh, 1978; Tiwari, 1972). Among organic source of nitrogen, urea has been found to support growth of certain cyanobacteria (Sings, 1985). Various amino acids, amides and amino acids mixtures can serve as a source of nitrogen for other strains (Carr and Whitton, 1982; Syrett, 1988; Davies, 1988; Mak and Trevan, 1988; Thomas *et al.*, 1975).

The cyanobacterial photosynthetic apparatus is located in photosynthetic thylakoids and consists of photosystem I (PS I) and photosystem II (PS II) interconnected with photosynthetic electron transport chain comprising plastoquinone, cytochrome *b/f* and plastocyanin. The characteristic accessory photosynthetic pigment of the group is predominantly phycocyanin, although in some forms both phycocyanin and phycoerythrin are found, where they play an indispensable role in chromatic adaptation (Kallas, 1994).

The majority of cyanobacteria are diazotrophic capable of carrying out simultaneously two incompatible reactions viz. oxygenic photosynthesis and O_2 sensitive N_2-fixation. In addition, they exhibit photo-production of hydrogen and photo-assimilation of nitrate, CO_2 and sulphate. The uniqueness of diazotrophic cyanobacteria lies in the fact that they are never energy limited and hence can continue producing H_2 from H_2O and NH^+_4 from N_2 and NO_3^- (Flores and Herrero, 1994) Their ability to grow over a wide range of ecological situations such as light, temperature, salinity, alkalinity and pollution makes them a model photosynthetic prokaryote and study the fundamental process that participates in regulating their adaptation to varying degree of ecological amplitudes. In other words, they must have developed an information processing system that enable them to

cope with changing environmental conditions and thus make them survive and grow in the given situations.

2.1.2. NITROGEN METABOLISM:-

Cyanobacteria are a diverse and widely distributed group of prokaryotes which are boond to carry out oxygenic photosynthesis, making a large contribution to global nitrogen economy by unicellular, filamentous non-heterocystous and filamentous heterocystous form (Stanier and Cohen-Bazire, 1977; Haselkorn, 1978; Bothe *et al.*. 1980; Gallon, 1980; Fay, 1992).

Nitrogen constitutes as much as about 10-11% of the total dry, weight of an exponentially growing cyanobacterial cell (Wolk, 1991; Flores and Herrero, 1994; Stal, 2000). Availability of nitrogen has been identified in many instances as the factor limiting productivity in natural habitats. Cyanonbacteria mainly use the inorganic species of nitrogen such as dinitrogen, nitrate and ammonium, in decreasing order of availability. In general, they can use nitrate or ammonium as the sole nitrogen source for growth, and many have additional ability to fix N_2. However, urea and other organic sources of nitrogen such as some amino acids, can also be assimilated by some cyanobacteria (Tandeau de Marsac and Houmard, 1993; Singh and Bisen, 1993, 1994, 1994b, d; Flores and Herrero, 1994; Reyes *et al.*, 1997; Stal, 2000).

Under autotrophic conditions, the utilization of any form of nitrogen by cyanobacteria is strictly dependent on light and CO_2 requirement for reductants and ATP in the assimilatory process

involved. The CO_2 requirement results from a set of interactions between carbon and nitrogen metabolism, some of which are regulatory in nature and determine that the assimilation, of carbon and nitrogen arc balanced.

The fact that inorganic nitrogen sources other than NH_4^+ are first metabolized to NH_4^+ prior to assimilation of their nitrogen atoms into nucleic acid, cell wall, porphyrins, polyamines and proteins. Some amino acids, like arginine, are assimilated by the production of NH_4^+ among their catabolic products. *Glutamine synthetase* and *glutamate synthetase* (GS/GOGAT) play a central role in cyanobacterial nitrogen metabolism particularly in NH_4^+ assimilation. Glutamate produced in GS/GOGAT pathway is not only the major nitrogen donor for the biosynthesis of other nitrogen containing metabolites but is itself a precursor for some amino acids and 5-aminolevulinate, the immediate precursor for porphyrin, phycobilin and chlorophyll biosynthesis. Glutamine also donates nitrogen to some metabolites. It is evident that amino acids arginine and aspartate together make up cyanophycin, a unique storage reservoir of nitrogen (and carbon) in many cyanobacteria.

Utilization of different nitrogen sources requires their entry through the cell wall with the aid of specialized transport system followed by their hydrolysis reduction. Nitrogen-fixing cyanobacteria possess a central position in the nutrient cycling largely due to their inherent capacity to fix atmospheric nitrogen directly into ammonium, making it available to higher plant (Kumar *et al.,* 1996). According to one assumption cyanobacteria fix over 35 million tones of nitrogen annually (Hader *et al.,* 1989; Kumar *et al.*, 1996).

2.1.3. CELLULAR-ORGANIZATION:-

The general cellular organization of the cyanobacterial cell is prokaryotic. The cell possesses following characteristic features (Fogg *et al.,* 1973; Beesley, 1994):

i) The photosynthetic pigments are located in thylakoids, which lie free in the cytoplasm, instead of being enclosed in chloroplasts as in photosynthetic eukaryotes.

ii) The thylakoids contain chlorophyll *a*, but chlorophylls *b* and *c* are absent.

iii) A diversity of characteristic cytoplasmic granular inclusions.

iv) Cell wall consists of murein (peptidoglycan), as in other eubacteria, outside which there is a lipopolysaccharide layer.

v) A fibrous sheath.

In some species of cyanobacteria (e.g. *Synechococcus lividus*), the protoplast can be divided into two distinct regions: a peripheral pigmented region called the chromatoplasm, containing the photosynthetic apparatus, and a central region termed as the centroplasm, containing DNA fibrils (Fogg *et al.,* 1973; Beesley, 1994).

The photosynthetic apparatus is similar to that in higher plants where the two photosystems (PS I and PS II) are situated within flattened vesicles called thylakoids, which are located throughout the outer regions of the cyanobacterial cell (Stanier and Cohen-Bazire,

1977; Fujita *et al.,* 1994). While, there is much evidence to support the endosymbictic theory that cyanobacteria were the progenitors of plant chloroplast (Shilo, 1982; Muro-Pastor *et al.,* 1999). Cyanobacteria do not contain the chlorophyll *b* pigment, which harvests light in photosynthetic eukaryotes. Instead they contain unique multimolecular structures known as phycobilisomes. These are situated on the outer surface of the thylakoids and are comprised of phycobiliprotein sub-units each containing an ordered array of covalently attached bilin prosthetic groups and linker polypeptides (Stanier and Cohen-Bazire, 1977; Sidler, 1994). Phycocyanin and phycoerythrin are the two most common bilins and they efficiently funnel more than 95% of the excitation energy from light to the reaction centre of PS 11 (Glazer, 1989; Sidler, 1994).

Cyanobacteria contain a large variety of intracellular inclusions, some of which are likely to serve as reserves of metabolites for use in conditions of nutrient imbalance (Fogg *et al*., 1973; Fuhs, 1973; Allen, 1984; Jenson, 1984, 1985a, b). For example, cyanophycin granules are high molecular mass proteinaceous granules consisting of non-ribosomally synthesised polymers containing equimolar quantities of arginine and aspartate; the arginyl residues are attached to all the free carbonyl groups of a polyaspartic acid core (Simon and Weathers, 1976). Thus, cyanophycin can serve as a source of nitrogen and carbon (Miller and Lang, 1968; Simon, 1973). Polyglucose (glycogen bodies) and poly β-hydroxybutyrate granules (in *Chlorogloea)* are reserves of carbon and a source of energy, while polyphosphate bodies are stores of phosphate (Harold, 1966; Cohen and Paras, 1976).

Carboxysomes are polyhedral bodies containing carbonic anhydrase and ribulose-1, 5-biphosphate carboxylase oxygenase (Rubisco, the key enzyme required for the net assimilation of inorganic carbon), as well as a few additional enzymes related to carbon metabolism. They are the major sites of carbon-fixation (Shively, 1988), except for carboxysomes. However, little attention has been paid to the synthesis and role of these inclusions. They may increase the chance of survival in a changing and possible stressful environment (Tandeau de Marsac and Houmard, 1993).

2.1.3.1. VEGETATIVE CELLS:-

Vegetative cells have both photosystems (PS I and PS II) similar to the higher plants. The photosynthetic pigments are located in thylakoid in the outer regions of the cells. In addition to chlorophyll a (chl *a*), carotenes and xanthophylls, they have characteristic accessory pigments called phycobiliproteins (Patterson, 1996).

They have a role as light receptors, but appear to function also as readily usable nitrogen store. Vegetative cells may also contain polyhedral bodies called carboxysomes. These are molecular aggregates of the calvin-cycle enzyme, ribulose biphosphate carboxylase/oxygenase (Rubisco). The presence of rubisco in vegetative cells and its absence from heterocyst was shown first time by using immunogold localization technique (Rai *et al.*, 1989). While heterocysts import the fixed carbon from vegetative cells, polyglucoside bodies may be found among the thylakoids. Excess nitrogen is stored in structured granules of copolymers of aspartic acid

and arginine known as cyanophycin. In addition, vegetative cells may also contain gas vacuoles, polyphosphate and lipid bodies. Thus, they are well equipped to accumulate essential metabolites.

2.1.3.2. HETEROCYSTS:-

Heterocysts are differentiated cells and the site of nitrogen fixation in cyanobacteria. In filamentous cyanobacteria, heterocyst differentiate from vegetative cells which have lost the capacity of oxygenic photosynthesis and have evolved a modified thick cell envelop (Singh *et al.,* 1999; Adams, 2000; Yoon and Golden, 2001).

The development of heterocysts is perhaps one of the most important characteristics of cyanobacteria because no other prokaryotes possess an equivalent degree of differentiation (Whitton and Carr, 1982; Wolk *et al.*, 1994; Singh *et al.*, 2000).

Heterocyst usually develops only when the concentration of combined nitrogen source is lowere I (Foggs, 1944; Castenholz and Waterbury, 1989). Heterocysts are terminally differentiated cells that develop in a pattern from certain vegetative cells in the filament in response to limitation in fixed nitrogen (Wolk *et al.,* 1994; Thiel, 1996).

Nitrogen fixing cyanobacteria become more important as the availability of combined nitrogen in the environment becomes a limiting factor for growth. The thick cell wall of heterocyst contains special lipopolysaccharides and forms a diffusion barrier for gases, limiting the entry of oxygen is evolved in these cells. Therefore, the

heterocyst is virtually anoxic and provides an excellent environment for the oxygen sensitive nitrogenase. PS I mediated conversion of light energy in the heterocyst provides ATP to nitrogenase. However, for reducing equivalents nitrogenase depends on the transportation of carbohydrate from the neighbouring vegetative cells. Nitrogen fixing cyanobacteria have a major advantage of times when source of combined inorganic nitrogen have been depleted from the water. Cyanobacteria have high competitive ability for ammonium under nitrogen limiting conditions, but a low competitive ability for nitrate in comparison with eukaryotic algae.

2.1.3.3. AKINETES:-

Akinetes are large thick walled cells, full of reserve materials, which enable cyanobacteria to survive when environmental conditions are not favourable for growth (Janczewski, 1874; Bornet and Flahault, 1886; Bristol, 1919; Lipman, 1941; Yamamoto, 1975; Whitton, 1992). They are much larger than vegetative cells, although their size can vary, even within a single culture (Clark and Jenson, 1969; Wolk, 1965, 1973) and its cytoplasm is more granular than the vegetative cells (Wildon and Mercer, 1963; Branue, 1980). They also and lack the photosynthetic pigments (Gomont, 1888; Fritsch, 1905). Fay (1969) found that chlorophyll was largely replaced by pheophytin and contained larger proportion of xanthophylls than the intact filaments.

The akinetes contain four times as much RNA as the vegetative cells with few exceptions as well as protein content was also three times higher than vegetative cells (Simon, 1977). Fay (1987) suggested

that the increased endogenous respiration in isolated akinetes indicates the reorganisation of enzyme activity prior to germination.

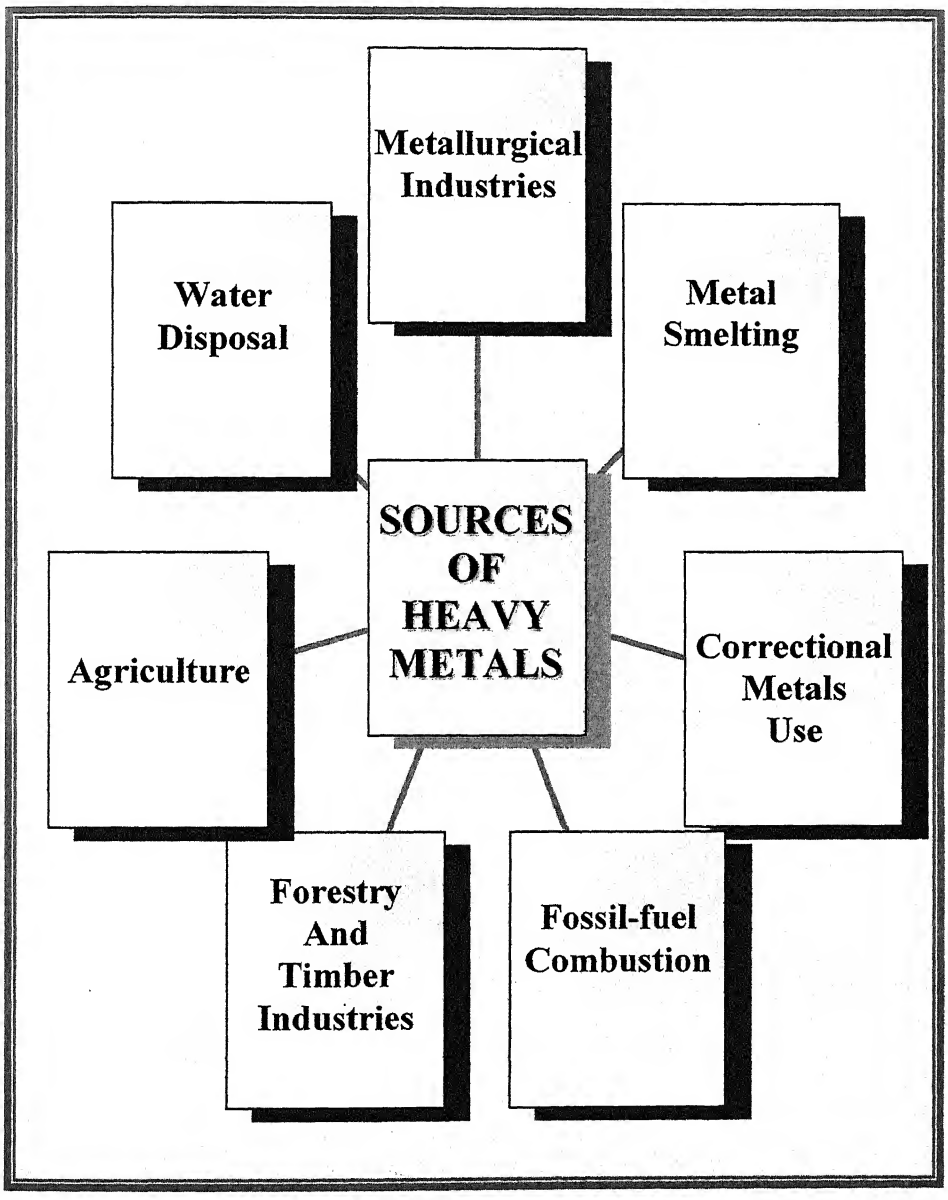
A deficiency of fixed nitrogen (Harder, 1917), iron (Sinclair and Whitton, 1977) and phosphate (Wolk 1965; Gentile and Maloney, 1969) with exception of planktonic species are reported to induce akinetes formation. Later on, it was observed that addition of nitrogen (nitrate, nitrite and ammonia) inhibited the heterocyst differentiation induced akinetes formation in *Anabaena* and *Nostoc* 7524 (Sutherlend *et al.,* 1979). Sodium glutamate sodium chloride and various amino acids (Tryptophan, aspartic acid, phenylamine, praline and isoleucine) have been shown to induce akinetes formation (Hirosama and Wolk, 1979a). While the addition of calcium gluconate resulted in the formation of longer chain of akinetes (Wolk, 1965) with *cylindrospermann licheniforme* kutz as exception in which only increase in akinetes production was reported (Hirosama and Wolk, 1979b).

Akinetes do permit survival of the organisms under adverse conditions, although they are not always necessary since many strains (including those capable of producing akinetes) can survive in the vegetative state. They certainly ensure the long term survival of the organisms. Livingstone and Jaworskii (1980) isolated viable akinetes from sediments as old as 64 years.

2.2. HEAVY METALS:-

The term heavy metal refers to any metallic chemical element that has a relatively high density and is toxic or poisonous at low concentrations. Examples of heavy metals include mercury (Hg), cadmium (Cd), copper (Cu), arsenic (As), chromium (Cr), thallium (Tl), and lead (Pb). The term heavy metal has generally been used to describe those metals having an atomic number greater than iron or having a density greater than iron or having a density greater than 5 g ml^{-1} (Passow *et al.,* 1961; Sorentino, 1979) but in current view, classification of metals based on their biological activity to bind with legends like S, O, Cl and most of the toxic metals fall under the category "borderline elements". Heavy metals are reported to cause various effects in algae depending upon the nature of particular heavy metal and its concentration. The algae show a visual symptom such as depression of net growth rate, morphological changes in cells and eventually the death of algae. Low concentrations of molybdenum, manganese, copper and iron have been demonstrated to be essential nutrient for all algae. Vanadium, cobalt and zinc are necessary for healthy growth and reproduction of some species (Noda and Horiguchi, 1971). Some metals can be essential at low concentration but may be toxic at higher levels such as Fe, Mn, Cu, B, Zn etc. but other metal ions above a certain concentration adversely effect on growth and metabolism known as non-essential elements. The term heavy metal refers to all those metals having a density greater than five. These includes As, Cu, Cd, Zn, Cr, Hg, Al, Ni, Co, Pb and Mn etc.

Figure: B Diagrammatic representation of different sources of heavy metals



2.3. HEAVY METAL TOXICITY:-

Heavy metals have a high affinity to sulphur metabolism and its effects are on *ATP sulfurylase* and *adenosine 5' phosphosulfate sulfotransferase* (Nnussabaum *et al.,* 1988). Once the heavy metals entered the cytosol, another system strictly related to sulphur metabolism is promptly activated, finally resulting in the production of important complexing agents, termed phytochelatins, which may contribute decisively towards rendering the metal ineffective. Prolonged *phytochelations synthase.* Phytochelatins were isolated and purified for the first time by Grill *et al.,* (1985). Heavy metals are able to activate the synthesis of phtochelatins having the following general structure $(\gamma$- Glu-Cys$)_n$–Gly, where n = 2 to 11. Phytochelatin form various complexes with various metals, due to presence of the thiolic groups of Cys, which chelate the heavy metals and as a result prevent it from circulating as metal ions inside the cytosol. Heavy metals are natural components of Earth's crust. They cannot be degraded or destroyed. To a small extent they enter our bodies *via* food, drinking water and air. Heavy metals are dangerous because they tend to bioaccumulation. Heavy metals can enter in water supply by industrial and consumer waste, or even from acidic rain, breaking down soils and releasing heavy metals into streams, lakes, rivers and groundwater.

Heavy metal toxicity was first identified as field problem by Gravil (1998) who described the toxic effect in plants growth in lead polluted area in Britain. The direct short-term effects of heavy metals on cyanobacteria have been subjected to different types of laboratory studies (Singh *et al.,* 1999; Rangasayatorn *et al.,* 2006). However, the

secondary or indirect effects of heavy metal on algae that is the change in the structure and function of algal communities resulting from toxic effects on the other organism of aquatic community (Bryant., 1994; Abou-Waly *et al.,* 1991).

Technological advancements have resulted in pollution world over. Out of the solid, liquid and gaseous wastes; and liquid waste could be effectively and economically treated by biological means. In stabilization pond, unlike bacteria, cyanobacteria are ideal since they are oxygen evolving photosynthetic prokaryotes bringing about oxygenation and mineralization and need only a small land area. They appear as ideal organisms because of their wide adaptability to varied environment, ability to degrade and metabolize varied compounds and their high utilization potential. Cyanobacteria have been used to remove heavy metal from aqueous system since they have high capacity to accumulate dissolve metal (Volesky, 1995; Ting *et al.,* 1989). The suspended and immobilized cultures of micro-algae shown that their cell response depends mainly on the metal (types, concentration and activity), on the species used. Immobilization process may increase tolerance to heavy metals (Lee *et al.,* 1999). Studies have been suggested that hydrogen ions play an important role in multi-component sorption because metal removal forms the aqueous is significantly affected by the initial pH of solution (Les and Walker, 1984). Cadmium uptake by cyanobacteria increased with increasing pH of solution (Rangasayatorn *et al.,* 2006). Micro-algae show certain attraction for polyvalent ions resulting on the removal of heavy metals from the environment.

Heavy metals in general, are known to inhibit *NADP-oxidoreductase* (Fisher and Frood, 1980), solublise cell membrane (Rai *et al.,* 1981) inactivate enzymes (Eichorn, 1974; Pickett *et al.,* 1975) reduce the supply of electrons for photo-phosphorylation (Bradeen *et al.,* 1973) transport of carbon compounds, synthesis of photosynthetic pigments (De Filippis and Pallaghy, 1976) and the supply of ATP and reductants leading to inhibition of photosynthesis and *nitrogenase* activity (Stratton *et al.,* 1979).

Ni was found to be more toxic against growth, pigments and heterocyst differentiation compared to the other metals. Inhibition of pigments and heterocyst differentiation compared to the other metals. Inhibition of pigment showed the following trend: Chlorophyll greater than phycocyanin and phycocyanin greater than carotenoid. The loss of K^+ and Na^+ as affected by Cr, Ni and Pb was similar but more pronounced for K^+ and Na^+. The inhibition of physiological variables depicted the following trend: Na^+ loss greater than K^+ loss greater than *glutamine synthetase* greater than NH_4^+ uptake greater than NO_3^- uptake greater than *nitrate reductase* greater than heterocyst frequency. Study therefore suggested that loss of electrolytes could be used as a final signal of metal toxicity in cyanobacteria (Rai *et al.,* 1990).

Toxicity of chromium and tin on growth, heterocyst differentiation, *nitrogenase* activity of *Anabaena doliolum* and its amelioration by S-containing amino acids and thiols has been studied by Rai and Dubey (1989). The final growth yield was found to be approximately 51% and 58% of control at sub-lethal concentration of chromium and tin respectively. Some interacting cations, viz. Ca, Mg, and Mn, substantially antagonized the toxic effect of chromium and tin

with reference to growth and nutrient (NO_3^- and NH_4^+) uptake in the sequence of hierarchy was Mg greater than Ca for *nitrate reductase* and *glutamine synthetase* activity of *Anabaena doliolum*, when Ni, Co and Zn were bivalent cations followed the synergistic inhibition sequence Ni greater than Co greater than Zn and potentate the toxicity of test metals in the Nitrogen fixing cyanobacterium.

Heavy metals play an important and vital role in biological reaction, as they activate numerous key enzymes and help in the stability of infrastructure of the body. We shall learn bellow that they're certain (20-30) metals that are essential for animals (vertebrates), and the rest non-essential. However, both essential (if in access), and non-essential heavy metal cause various disorders, some being incurable in latter stage. For instance cobalt and manganese, both essential heavy metals respectively cause hepatic-renal disorders and maganica locura, a crippling neurological disorder similar to Parkinson's disease. Besides these diseases, there are many other diseases such as minimata, itai-itai, bush sickness, Wilson's disease, white muscle disease, genu-velgum disease etc. that are attributed to heavy metal toxicity.

2.4. BIOREMEDIATION:-

Cyanobacteria are unique group of nitrogen-fixing and oxygen evolving photoautotroph showing high degree of morphological and developmental complexicity (Dwivedi *et al.,* 2004). Besides their presence in natural systems, they are abundant in rice fields and have potential source of nitrogenous biofertilizer input to agriculture without

making any use of fossil fuels. They help to enhance agriculture productivity by adding fixed nitrogen and extracellular products to paddies crop (Venkataraman *et al.,* 1975). Cyanobacteria are taxonomically diversified; they are unicellular, colonial and filamentous with false branching, basically they are divided in two groups: (i) heterocystous and (ii) non-heterocystous. Heterocystous cyanobacteria are well recognized due to N_2 fixation and their importance in improving soil fertilely for suitable agriculture in submerged and irrigated rice cultivation (Venkataraman, 1992); while previously non-heterocystous strains have been an encountered group due to absence of special cell known for N_2 fixation and simple cellular organization but now-a-days some reports indicated that these forms i.e., *Oscillatoria, Microcoleus, Plectonema, Porphyrosiphon, Lyngbya,* and *Trichodesmium* have been capable to fix atmospheric nitrogen under anaerobic and micro-aerobic conditions (Tiwari *et al.,* 2000) and they also possess remarkable tolerance to the biocides, viz., 2,4-dichlorophenoxy acetic acid, malathion and dimecron at doses much higher than those recommended for rice crops. The strain of *Anabaena* could tolerate 100 ppm Ceresan M (N-ethylmercuri p-toluene sulphonilide) while strain of *Tolypothix tenius* have been found sensitive up to a concentration of 0.1 ppm Ceresan. Effect of different concentrations of Malathion (10-200 ppm, v/v) on the growth and chlorophyll *a* content of *Nostoc linckia* and *Westiellopsis* sp. have been reported that growth of both the cyanobacteria decreased with increased concentrations of Malathion, however both the species could tolerate up to 150 ppm (v/v) of malathion concentration, lethal dose of the pesticide was 200 ppm (v/v) for the cyanobacterial strains. In *N. linckia* the chlorophyll *a* content decreased in lower concentration (10 ppm, v/v) while in *Westiellopsis* sp. chlorophyll-*a* was higher then the

control value. A survey of rice fields and in fresh water ecosystem especially in India showed that the non-heterocystous cyanobacterial population was much higher than the population was much higher than the population of heterocystous strain (Tiwari *et al.,* 2000; Dwivedi *et al.,* 2004). A total number of 12480 algal species of different groups reported from India, out of which cyanophycean forms belonging to 100 genera have been reported from different parts of Indian subcontinent in which 422 species found in marine habitats.

Cyanobacteria are the largest and most diverse group of photosynthetic prokaryotes. Their habitats vary from fresh water to terrestrial environment. They are oxygen-evolving organisms that respond stress conditions such as light deprivation. Heavy metals are ubiquitous in the biosphere where they occur as a part of the natural constituents of chemicals to which biota and human beings are frequently exposed. This results in introduction of substantial amounts of potentially toxic metals into the food chain. Microorganisms from a group of inseparable interacting communities, subjected to such unfavorable alteration of the aquatic and other ecosystems. Cyanobacteria, a group of prokaryotic, photosynthetic nitrogen fixers are present in every ecological niche and therefore exposed to the toxic effect of the metals have been studied with respect to growth, *nitrogenase* activity and carbon fixation. (Ting *et al.,* 1990;Cano *et al.,* 1993; Blackwell *et al.,* 1995; Chaun and Liu.,1996; James *et al.,*1996; Bhome *et al.*,1998; Barman *et al.*,2000; Armienta *et al.*,2001., Epniewska and Bucior.,2001; Suresh Babu *et al.*,2001). These cells have developed natural methods of responding to metals such as copper, lead, and cadmium though passive accumulation in cells and though surface binding to various functional groups. They have also

been found to remove harmful metals from the environment. For example, *Spirulina platensis,* shown to contain detectable levels of mercury and lead when grown under contaminated conditions, implies that this cyanobacterium was taking up the toxic metal ions from its environment. Further studies confirmed that this cyanobacterium both adsorbs and takes up metal ions. Reports also indicate that carboxyl groups on algal biomass are responsible for binding to various ions.

Several reports towards applying cyanobacteria using pollution abetment have been demonstrative (Mohamed, 2001; Tien, 2002). Recently certain cyanobacteria such as *Lymgbya spiralis* and *Phormidium molle* found to accumulate Hg 96%, 94%, 94% and 93% respectively (Inthorn *et al.,* 2001) who also reported the cyanobacterial strains; namely *Nostoc punciforme, Oscillatoria agardhi, Gloecapsa, N. piscinale, N. commune* and *N. paludom* removed lead from the aqueous environment 98%, 96%, 96%, 94%, 94% and 92% respectively. Barbara Pawlik and Skowronska, (2002) found the correlation between toxic lead effect and production of lead induced thiol peptides in the microalga *Stichococcus bacillaris.*

The ubiquity of heavy metals in the biosphere results in the introduction of high amount of toxic metals into the food chain from various sources. (Meenakshi Banerjee *et al.*, 2004). The recovery of valuable toxic metals by biological methods, attractive alternation to conventional physiochemical process is finding significant application in various remediation strategies. (Beverridge *et al.*, 1989; Ehlich *et al.*, 1990; Geddie *et al.*, 1993; Lester *et al.*, 1984; Scott *et al.,* 1988; Singh *et al.*, 1989; Volesky *et al.*, 1995). Metal sequestration by organism is

often termed biosorption, which includes both passive adsorptions of metals to cell envelopes a metabolically mediated uptake (Campbell and Smith, 1986; Geesey and Jang, 1989; Johanson and Shubert., 1986; Rai *et al* 1992).

Nostoc muscorum can be protected from Cr and Pb toxicity by supplementing 10µg ml^{-1} of ascorbic acid, reduced glutathione, and 5µg ml^{-1} of sulphur containing amino acids (L- methionine and L-cysteine). Appreciable stimulation of different parameters of *Nostoc muscorum* by ascorbic acid and glutathione was noticed in the untreated culture. These substances when used in the presence of metals were found to restore ionic loss, growth, heyterocyst production, and *nitrogenase* activity thus acting as reducing agents. S-containing amino acids (at 5 microgram ml^{-1}) exert little stimulatory effect on untreated cultures. These results suggest that sulfer-containing compounds and reducing agent can protect against metal toxicity possibly by providing a reducing power which protects the enzymes from undergoing oxidation. Inhibition of heterocyst frequency and *nitrogenase* activity by L-methionine and L-cysteine suggests that these amino acids possibly act as a nitrogen source. Study clearly suggested that metal toxicity in nitrogen fixing cyanobacterium may be appreciably counteracted by reducing substances and amino acids which are likely to occur in natural habitats.

Recently, Naoto Yoshida *et al*., (2005) of Miyazaki University, Japan; have been isolated unicellular algae, *Chlorella sorokiniana* from soil displaying a high growth rate under heterophilic conditions. The optimum temperature for growth was 35°C and the optimum pH was 6.0 to 7.0. Glucose, sucrose, galactose, maltose and soluble starch

served as carbon sources supporting growth under dark conditions. The cell yield was 50 gm l^{-1} in a heterophilic medium containing 3% glucose. Isolated unicellular algae were highly resistant to heavy metals such as cadmium, of which the minimal inhibitory concentration was 4mM. Algae were capable of taking up the heavy metal ions Cd^{+2}, Zn^{+2}, and Cu^{+2} at 43.0, 42.0 and 46.4µg mg^{-1} dry weight respectively. These results indicated that isolate was potentially useful for bioremediation by preventing environmental dispersion of heavy metals.

2.5. ROLE OF CYANOBACTERIA IN ENVIRONMENTAL MONITORING:-

Cyanobacteria are excellent models for toxicity studies because as photoautotroph and primary producers deleterious on them will have implications for higher organism in the food chain (Lee *et al.,* 2002). As the algae are good indicators of water pollution and they are increasingly used in phycotoxicity tests for environmental monitoring (Ting *et al.,* 1991) and they are also capable of accumulating heavy metals to concentrations several orders of magnitude higher than the surrounding medium (Becker, 1986; Taneja and Fatima, 2000). In this context, numerous studies have been conducted and concluded that several cyanobacterial strains were found to be potential accumulator of heavy metals and the preferential sequence of metals accumulation varies from species to species. According to Rai *et al.,* (2000) *Anabaena doliolum* was found to be potential accumulator of heavy metals and accumulate the metals in an order of Cu, Ni, Fe, Mn and Zn from the fly-ash and also enhance about ten fold nitrogen content of fly-ash by nitrogen fixation. It also increases available phosphorus

percentage in fly-ash. They also tested some other cyanobacterial strains viz. *Nostoc calcicola, N. commune, Aulosira fertillisima* and *Sycctonema oscillatum* for metal tolerance and found only two species viz. *N. calcicola* and *N. commune* show average growth while rest of the species do not grow on fly-ash under controlled conditions. The cyanobacterial bloom consisted of *Microcystis deuginosa* that accumulated heavy metals. Since the lake water was not contaminated with chromium, the traces of chromium accumulated by the cyanobacteria may be used as an early warning system for chromium contamination in the lake (Ali *et al.,* 1999). They further advocated that the blooms of *M. aeruginosa* may prove hazardous to the consumer in view of public supply. Additionally, presence of *M. aeruginosa* in lake water may prove hazardous due to its disease (Rhinosporidiosis) causing potential posing health problems to human being using lake water.

Many heavy metals such as copper, nickel and zinc are well known as essential trace elements for cyanobacteria and plants. But the beneficial range of heavy metal in particular copper, concentration is extremely narrow, so that even the slightly elevated natural concentrations in part of the oceans are toxic to some cyanobacteria. Above the threshold value heavy metal leading to growth inhibition, a variety of toxic effects have been observed in different environmental conditions including irradiance, the length of the light phase and pH of the medium. Many of these effects, however, have only been analyzed *in vitro,* and some could not be verified by later *in vivo* or *in vitro* studies. One such mechanism is the substitution of the central ion of chlorophyll, magnesium, by heavy metals. The resulting heavy metal

chlorophyll leads to a breakdown of photosynthesis in a variety of ways.

Wilde and Benemann (1993) concluded that biosorption of heavy metals has the potential to be more efficient and cheaper that of the quaternary waste water treatment processes. *Spirulina* have been found to be more potential, among the other cyanobacteria such as *Chlorella, Sceedesmus, Chlamydomonas* (Bedell and Darnall, 1990). Recently, Lee and Lustigman (2000) stated that *Anacystis nidulans* have been very sensitive to combinations of mercury and selenium and inhibitory concentration of SeO_2 was 50 mg l^{-1} $HgCl_2$, while that of 5 mg l^{-1} $HgCl_2$ was toxic. Mercury treated cells were found smaller in size that the control (Lee *et al.*, 1999, 2000). Lustigman *et al* (2000) have been determined the thallium effect on the growth of *A. nidulans* and found the cells were smaller, more slender and pale at 5 mg l^{-1} $TiNO_3$ while at 10 mg l^{-1} and higher, they were colorless. Thus now day's prokaryotic algae are regarded as relevant indicator in the field of environmental monitoring and assessment to a number of pollutants they are frequently utilized in eco-toxicological screening of contaminated fresh and marine water. Recently, Rangsayatoron *et al.*, 2006 (Mahidol University, Thailand) used *Spirulina platensis* to remove low concentrations of cadmium (less than 100 mg l^{-1}) from fresh water. The cyanobacteria were exposed to 6 different cadmium concentrations for 96 h, and the growth rate was determined using an optical density at 560 nm. The inhibitory concentration was estimated using probit analysis. The inhibiting concentrations IC [50] at 24, 48, 72, and 96 hours were 13.15, 16.68, 17.28 and 18.35 mg l^{-1} Cd respectively, Cellular damage was studied under a light microscope. Swollen cells and fragmented filaments were observed. Cell injury

increased with increasing concentrations of cadmium. Ultra-structural changes were observed in the algae exposed to cadmium concentrations both close to IC [50] (14.68 mg l^{-1}) and at IC [50] (18.35 mg l^{-1}). The alterations induced by cadmium were disintegration and disorganization of thylakoid membranes, presence of large intra-thylakoidal space, increase of polyphosphate bodies and cell lysis. In addition the cadmium adsorption by algal cells was studied. Environmental factors were found to have an effect on biosorption. The uptake of cadmium was not affected by temperature of the solution, but the sorption was pH dependent. The optimum pH of biosorption of algal cell was 7. The cadmium uptake process was rapid with 78% of metal sorption completed within 5 minutes. The sorption data fit well to the Langmuir isotherm. The maximum adsorption capacity for *Spirulina platensis* was 98.04 mg Cd per gm biomass.

The biotransformation of mercury by cyanobacteria was investigated under aerobic and pH controlled culture conditions. Mercury was supplied as $HgCl_2$ in amounts emulating those found under heavily impacted environmental conditions where bioremediation would be appropriate. The analytical procedures used to measure mercury within the culture solution including that in the cyanobacterial cells used reduction under both acidic and alkaline conditions in the presence of $SnCl_2$. Acid reduction detected free Hg ions and its complex whereas alkaline reduction revealed that meta-cinnabar (β-HgS) constituted the major biotransformed and cellularly associated mercury pool. The nature of the biosorption depends on the metal and the microorganism. (De Filippis *et al.,* 1994; Gourdon *et al.,*1990 and Lester *et al.,* 1984).

2.6. METAL BINDING PROPERTIES AND DETOXIFICATION MECHANISM IN CYANOBACTERIA:-

Cyanobacteria are capable of accumulating metal ions by two well defined processes:-

(1). Biosorption "an energy independent binding of metal ions by cell wall" and

(2). Bioaccumulation "an energy dependent process of metal uptake by the cells".

Studies have demonstrated that the cyanobacteria are used for detoxification of environmental pollutants (Subramanian and Uma, 1996). Cyanobacteria play a vital role in the productivity of water bodies and utilization of atmospheric carbon di-oxide. The main cyanobacterial strains viz. species *Anabaena, Nostoc, Phormidium, Aphanocapsa, Oscillatoria, Lyngbya, Spirulina, Aulosira* and *Anacystis* appear to be promising bioremediators for the effluents rich in nitrates and phosphates. Though, there are reports on the use of cyanobacteria for the treatment of sewage and effluents. Cyanobacteria metabolize aromatic compounds though ring hydroxylation, methylation, side-chain hydroxylation, acetylation and formylation (Narro, 1985; Mc Eldowney *et al.,* 1993). It is demonstrated that *Aulosira fertillisma* and *Nostoc calcicola* able to degrade, detoxify and use the pesticides as their sole phosphate source though the production of a phosphate solubilizing enzyme (Subraminian *et al.,* 1994). The cyanobacteria produce siderphores that may responsible for absorption of metal ions. The metal accumulating cyanobacterial biomass can either be disposed

off or incinerated for metal recovery. It was also studied that cyanobacteria accelerate transformation and degradation of certain polycyclic aromatic hydrocarbons, organophosphorus compounds in the water by the sun light. Thus cyanobacterial detoxification of environmental pollutants could help in controlling the pollution of aquatic and terrestrial habitats.

Cyanobacteria have a cell wall or envelope that is capable of passively adsorbing high levels of dissolved metals, usually *via* a charge mediated attraction (Macaskie and Dean, 1990). Among these organisms some mucilage producing cyanobacteria were able to bind large amount of metals because mucilage consists of polysaccharides with smaller amount of protein. Since polysaccharides are the major components of this mucilage, it has been suggested that binging of heavy metals by these organisms takes place though these polysaccharides (Amemiya and Nakayama, 1984). The intracellular mechanisms involved are the binding of cations to legends analogues to *metallothionein* (Olafson *et al.,* 1980) in addition to the polysaccharides reserve (Jensen *et al.,* 1982; Singh *et al.,* 1992) further studies showed that *metallothionein* genes could be amplified by Cd in Cyanobacteria isolates from metal polluted aquatic environment (Singh *et al.,* 1999). According to Ren *et al.,* (1998) the synthesis of *metallothionein* (*MT*) can be induced by stress. Heavy metals such as cadmium, copper and mercury metals cause rapid de novo synthesis of *metallothionein* in the environment (Kojima, 1991). Thus it is interesting to introduce the *MT* gene into organisms for improving sequestration of toxic metals. Ren *et al.,* (1998) successfully introduced mouse liver *MT*-I into cyanobacteria to confer cadmium resistance and clear heavy metal pollution in water. Organization into –Cys-Cys-, Cys-X-Cys, or –Cys-X-X-Cys- sequence

(X corresponds to any other amino acid in the protein sequence). Synthesis of *metallothioneins* has been shown to be prompted by elevated concentrations of some metals (Turner and Robinson, 1995). *Metallothioneins* can be characterized into thee specific classes: class I contains most animal *metallothioneins*; class II consists of *metallothioneins* whose cysteine locations in the polypeptides are distantly related to those found in equine renal *metallothioneins*; and class III *metallothioneins* are characterized by atypical non-transisionally synthesized metal-thiolate polypeptides. Cyanobacterial *metallothioneins* belong to class II. They possess appropriately 56 amino acids including nine cysteine residues. This number of cysteine residues is less than the 20 cysteine residues out of 60 amino acids for the animal class I *metallothioneins* and 12 cysteine residues out of 75 amino acids for plant class I *metallothioneins*. Further tests were performed to investigate the abilitybto recover bound metals. *Synechococcus* PCC 7942 biomass was immobilized in a silica polymer matrix, then tested for its ability to bind copper (II), lead (II), nickel (II), and cadmium (II). Additionally, a genetic investigation was performed to identify genes for class II *metallothioneins* in *Synechococcus* PCC 7942.

The two mutant strains of *Nostoc calcicola* viz. *Met-R1* and *Met-R2* were found to remove the cadmium from the surrounding medium. *Met-R2* showed more than two-folds higher cadmium removal than the *Met-R1* strain (Ravendra *et al.,* 2002) while another cyanobacteria *Synechococcus* PCC 7942 and filamentous nitrogen fixing strain *Nostoc* PCC 7120 were found to accumulate the chromium from the $CrCl_3$ as a surrounding medium (Thomson *et al.,* 2002). The *Plectonema boryanum* is found to be extremely tolerant to zinc and can

be used as a indicator of zinc pollution (Rana *et al.,* 1971). *Westiellopsi* sp. has been studied by Taneja and Fatima (2000) and concluded that *Westiellopsis* tolerate 5 ppm copper concentration with the metal uptake capacity of 85.95 mg g^{-1} dw. The study suggested that it can be used as copper detoxifier / bioremedial agent for treating industrial effluent, sewage and other contaminated water bodies. The biosorption of heavy metals has the potential to be more efficient and cheaper than that of quaternary wastewater treatment processes. *Spirulina* sp. has been found to be more potential to remove heavy metals among the other green algae such as *Chlorella, Sceedesmus* and *Chlamydomonas.*

Cyanobacteria are capable of synthesizing on exposure to heavy metals, thiol-rich peptides (γ-glutamyl cysteinyl) n-glycine with n=2 to 11, also known as "phytochelatins". Phytochelatins are enzymatically synthesized by a specific *γ-glutamyl cysteine-dipeptidyl transpeptidase* (*phytochelation synthase*) that is activated by the presence of metal ions and uses glutathione as a substrate. Phytochelatins bind metal ions by thiolate coordination yielding intracellular metal complexes but in case of cyanobacteria they are capable of bind *metallothioneins*, sequester and buffer excess intracellular zinc. At present, the vast majority of the available experimental data relate to cyanobacterial *metallothionein*, *SMT*A, from *Synechococcus* PCC 7942. *SMT*A is required is required for normal resistance to zinc resistance has been used as a selectable marker. The imidazole groups histidine residues, in addition to thiol groups of histidine and cysteine residues co-ordinate zinc in bacterial *metallothioneins*. The structure of bacterial *metallothionein* must facilitate some discrimination between "adventitious" and "advantageous" zinc-binding sites such that under excess zinc conditions metal is predominantly scavenged from the former. It

remains unclear whether or not bacterial *metallothionein* also acts as a zinc store that supplies zinc-requiring proteins or if under some conditions it deactivates a subset of proteins via zinc removal. Expression of *SMT*A is induced in response to elevated concentrations of zinc via the action of *SMT*B. *SMT*B has some sequence similarity to the arsenic responsive repressor *Ars*R and genes encoding related proteins are present in many bacterial genomes. Metal perception by *SMT*B differs from *Ars*R. The latter contains a characteristic *Cys-Val-Cys* motif associated with a DNA-binding helix turn-helix (the *Ars*R motif), while the former contains metal-binding motifs associated with a carboxy terminal α-helix that form the interface between *SMT*B dimers (the *SMT*B motif). Some *SMT*B-*Ars*R family proteins, including the zinc sensor *Zia*R from the cyanobacterium *Synechocystis* PCC 6803, have the metal-sensory motifs of both *SMT*B and *Ars*R.

Live algae possess intra-cellular polyphosphates that participate in metal sequestration as well as algal extra-cellular polyphosphates that serve to chelate or bind metal ions. Strains of *Synechocystis* sp. have been shown to develop a thickened calyx when exposed to copper stressed growth conditions. *Synechococus* PCC 7942 was found to possess a copper transporting P-type *ATPase* in thylakoid membrane. *Synechococcus cedrorum* was shown to be tolerant to heavy metals and pesticides. Other investigators have studied the biosorption of heavy metals by algal biomass (Volesky and Holan., 1995). Such findings show the possibility of manipulating or over-expression existing resistance mechanisms and the use of such organisms to remove harmful metals from the environment. Studies showed binding of copper and nickel, and to a lesser extent lead, were favored at higher pH. This suggests that lowering pH may reverse binding.

1.7. EFFECTS OF HEAVY METALS ON CYANOBACTERIA:-

The continued discharge of heavy metals into the aquatic system though industrial, agricultural and domestic activities have significantly contaminated our aquatic resources. Increased circulation of toxic metals though water and their inevitable transfer to a higher trophic food chain is likely to cause important environmental hazards. The disastrous effects of heavy metals on phytoplankton have been well established with regard to eukaryotic algae (Rai *et al.,* 1981; Stokes, 1975; Whitton, 1970) and prokaryotic cyanobacteria Stratton *et al.,* 1979; Rai and Raizada, 1985). Although these metals have several functional roles, they may still adversely affect on enzymes or membranes of the cells, and disrupt biochemical pathways by forming complexes. Iron, nickel and copper required as micronutrient, are essential for various metabolic processes of cyanobacteria. Iron mediated changes in algal growth in Lake Eric, phytoplankton (*Anabaena, Scenedesmus* and *Slenastrum*) have been well documented by Vymazal (1990). Further Rai and Raizada (1986) confirmed that lower concentrations (10-50 mg ml^{-1}) of Ni were stimulatory for growth, carbon fixation, heterocyst differentiation and *nitrogenase* activity of *Nostoc muscorum.* The effect of toxic chemicals has been measured on a variety of algal species using different culture method and a number of different biological responses. The response of a particular species of algae to potential toxic contaminant is dependent on culture conditions. Many physical and chemical factors such as temperature, water hardness, pH, nutrient, toxic chemicals and heavy

metals effect on cyanobacteria. Effects of heavy metals on cyanobacteria are as follow: -

❖ PHENOTYPIC CHANGES:-

- ➢ Reduction or enlargement of cell size with changes in cell shape.
- ➢ Inhibition of heterocyst differentiation with enlargement of size.
- ➢ Inhibition of growth and survival rate.
- ➢ Inhibition of sporulation period with total spore forming frequency of algae.

❖ ULTRA STRUCTURAL CHANGES:-

- ➢ Degradation of polysaccharides layer of heterocyst.
- ➢ Degradation of peptidoglycan layer.
- ➢ Decrease in cyanophycean granules.
- ➢ Disorganization of thylakoid membrane.
- ➢ Increase of intrathylakoid space.
- ➢ Reduction of gas vacuoles.
- ➢ Increase in the number of polyphosphate bodies.

❖ BIOCHEMICAL CHANGES:-

- ➢ Inhibition of photosynthesis.
- ➢ Reduction in protein synthesis.
- ➢ Reduction in Nutrient uptake.
- ➢ Inhibition of Nitrogen fixation.
- ➢ Inhibition of *nitrate reductase* activity.
- ➢ Inhibition of carbon fixation.
- ➢ Breakdown of p- nitrophenyl phosphate (p-NPP).
- ➢ Inhibition of ATP synthesis.

CHAPTER – 3.

OBJECTIVES

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OBJECTIVES

- To find out minimum Cu concentration required for the optimum cell growth in free and immobilized cells of *Nostoc calcicola* Breb.
- To find out effect of Cu on various physiological and biochemical events in free and immobilized cells of *Nostoc calcicola* Breb.
- Comparison of Cu uptake in freely suspended and immobilized states of *Nostoc calcicola* Breb.
- To find out various reasons for more Cu transport in immobilized cells of *Nostoc calcicola* Breb.
- Isolation and characterization of Cu-tolerant (Cu^r) strain of *Nostoc calcicola* and to identify the mode of tolerance.

CHAPTER – 4.

MATERIALS AND METHODS

CHAPTER – 4.

MATERIALS AND METHODS

4.1. EXPERIMENTAL ORGANISM AND GROWTH CONDITION:-

Cyanobaceria are Gram negative prokaryotes that perform oxygenic photosynthesis like plants. These cells are important in increasing nitrogen fertility in paddy fields. *Nostoc calcicola* Breb, an isolate of rice field, obtained from Algal Research Laboratory, Centre of Advanced study in Botany, Banaras Hindu University, Varanasi (U.P.) INDIA, was mass cultured in a capacity of 500 ml Erlen-Mayer flask containing 200 ml Allen-Arnon's combined nitrogen free suspension medium at Biotechnology Research Laboratory, Department of Biotechnology, J.C. Bose Institute of Life Science, Bundelkhand University, Jhansi (U.P.) INDIA;. The free cells were sub-cultured in 50 ml Erlen-Mayer flask containing 100 ml Allen-Arnon's combined nitrogen free medium. The pH of the medium (8.0) was adjusted as adding NaOH or HCl before autoclaving. The cultures were incubated photoautotrophically in culture room (26 ± 1°C), which was illuminated by two cool fluorescent lights (Intensity 14.4 Watt m^{-2}; on the surface of culture vessels) with 18/6 light / dark cycle. All the cultures were routinely shaken (thrice in a day) with the help of rotator flask shakers at 130 rpm for 20-30 min. The axenic nature of the culture was checked periodically by standard microbiological techniques.

4.1.1. SYSTEMIC POSITION OF ORGANISM:-

Division : Cyanophyta
Class: Cyanophyceae
Order : Nostacales
Family : Nostocaceae
Genus : *Nostoc*
Species : *calcicola*
Subspecies : Breb

4.1.2. MORPHOLOGY OF *Nostoc calcicola* Breb:-

Nostoc calcicola Breb is free floating, filamentous, loosely entangled organism, forming thin, bright blue-green mass. Flagella are absent.

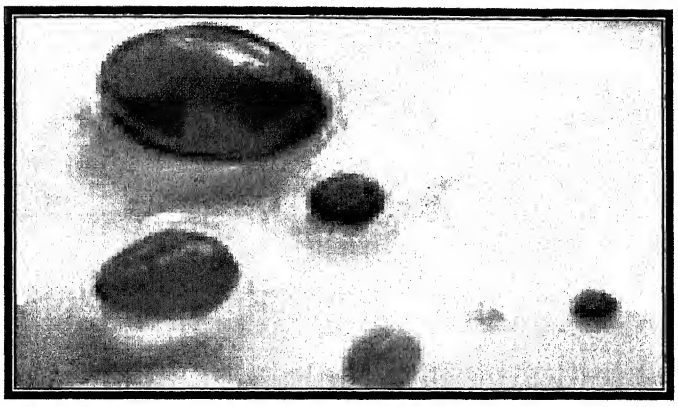
4.1.3. PHOTOSYNTHETIC PIGMENTS:-

Pigments of course constituents are of the primary characteristics of the cyanophyta. There is chlorophyll *a* (but not present chlorophyll *b*); phycocyanin *c*; phycoerythin and xanthophylls (allophycocyanin, zeaxanthin, mycoxanthophyll, asteroxanthin and lutin). The cell wall consists of an inner thin, cellulose layer, a medium Pectin layer and another mucilage layer. The cytoplasm occurs as chloroplasm.

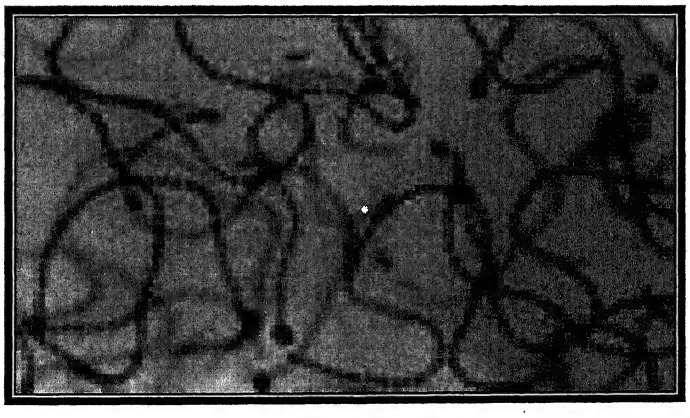
4.1.4. STORAGE PRODUCT:-

Cyanophycean starch, protein and amino acids are present as storage product.

PLATE : 1



(A)



(B)

(A) Colonies of *Nostoc calcicola* Breb on Agar medium.
(B) Filaments of *Nostoc calcicola* Breb.

COMPOSITION OF THE ALLEN-ARNON GROWTH MEDIUM

MACRONUTRIENTS: -

Sr. No.	Macronutrients	Concentration (mM)
(1)	$MgSO_4.7H_2O$	1.0
(2)	NaCl	4.0
(3)	$CaCl_2$	0.5
(4)	K_2HPO_4*	2.0

MICRONUTRIENTS: -

Sr. No.	Micronutrients	Concentration (mg l^{-1})
(1)	Fe-EDTA*	4.0
(2)	Mn ($MnSO_4.4H_2O$)	0.5
(3)	Mo (MoO_3)	0.1
(4)	Zn ($ZnSO_4.4H_2O$)	0.05
(5)	B (H_3BO_3)	0.05
(6)	$Co(Co(NO_3)_2.6H_2O$	0.01

* Autoclaved separately and added to the pre-cooled sterilized basal liquid medium to avoid precipitation.

EQUIPMENTS USED:-

- Atomic Absorption Spectrophotometer (Perkin Elmer, AAS-1450, USA).
- Autoclave (Jyoti Scientific Industries, Gwalior, M.P., India).
- BOD cum Shaker (Jyoti Scientific Industries, Gwalior, M.P., India).
- Electronic Weighing Balance (JA 3003 Precison Balance, India).
- Gas Chomatograph (Shimadzu, GC, Japan).
- Heating Mantle (Jyoti Scientific Industries, Gwalior, M.P.,India).
- Ultra Centrifuge (BIO-LAB, BL-165D; India).
- Laminar Air Flow (Ultra Scientific Industries, India).
- Liquid Scitillation Counter (Beckman, Model LS-7000, USA).
- Magnetic Stirrer with Hot Plate (Jyoti Scientific Industries, Gwalior, M.P., India).
- Microwave Oven (Bajaj 2800 ET-B; India).
- pH meter (Jyoti Scientific Industries, Gwalior, M.P., India).
- Rotatory Flask Shaker (Jyoti Scientific Industries, Gwalior, M.P., India).
- U.V.-Visible Spectrophotometer (UV-1700, Simadzu, Japan).
- Vortex mixer (Jyoti Scientific Industries, Gwalior, M.P., India).
- Water-Bath Shaker (Jyoti Scientific Industries, Gwalior, M.P., India).

CHEMICALS USED:-

- Acetone
- Alginic acid
- Ammonium molybdate
- ANSA (1-amino-2-nephthol-4-sulphuric acid)
- Aqueous phenol
- Boric acid
- Calcium chloride
- Copper sulphate
- Cobalt nitrate
- Chloroform
- Di-potassium hydrogen phosphate
- Ethylene diamine tetra acetic acid
- Feric Chloride
- Folin phenol Reagent
- Glutamine
- Hydrochloric acid
- Hydroxamine-HCl
- Imidazole HCl buffer
- Iron-containing ethylene diamine tetra acetic acid
- L-glutamate
- Manganese sulphate
- Magnesium Chloride
- Magnesium sulphate
- Methanol

- Molybdenum trioxide
- Nitric acid (concentrated)
- Perchloric acid
- Potassium Arsenate
- Potassium Chloride
- p-Nitrophenyl phosphate
- PPO (2,5-diphenyloxazol; Sigma, USA)
- POPOP {1,4 bis (4 methyl-5-phenyl-2 oxazol)-benzene; Sigma, USA}
- Sodium ADP
- Sodium carbonate
- Sodium chloride
- Sodium citrate
- Sodium hydroxide
- Sodium-potassium tartarate
- Sodium metabisulphite
- Sodium sulphite
- Sulphuric acid (concentrated)
- Trichloroacetic acid
- Tris-HCl Buffer
- Zinc sulphate
- ATP (neutralized salt)
- Toluene
- Trypsin (Sigma, USA)
- Trypsin inhibitor (Sigma, USA).

4.2. GENERAL PHOTOAUTOTROPHIC GROWTH:-

Growth was measured by the Quantitative Estimation of cellular protein content per ml algal culture, and specific growth rate constant (*K*) calculated by the formula as described by Kratz and Myers (1995):-

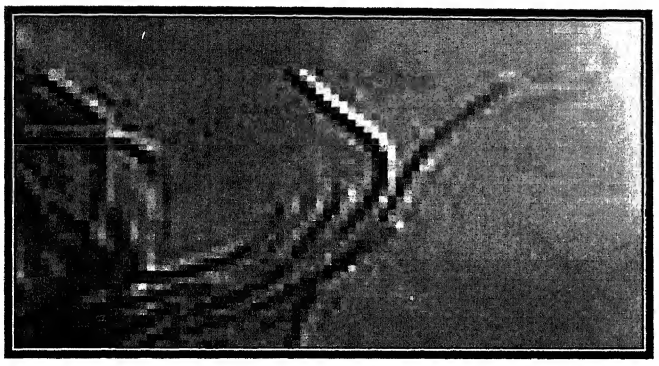
$$K = \frac{2.303 \text{ Log } N_2 - \text{Log } N_1}{T_2 - T_1}$$

Where N_1 is initial cell density at time T_1 and N_2 is the final cell density at time T_2.

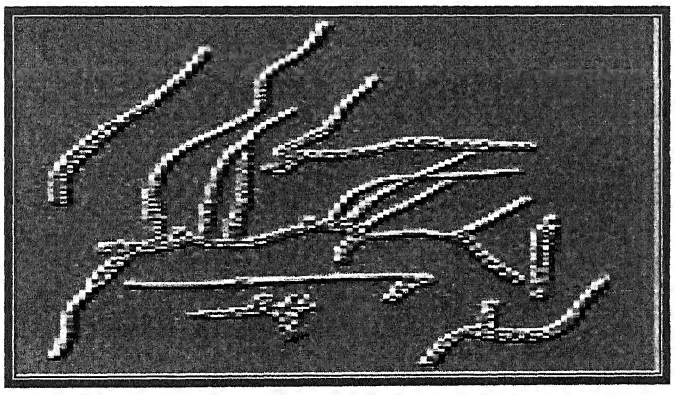
For maintenance of the algal strain, exponentially growing cyanobacterial cells (6-8 days old) were harvested by centrifugation (5000x g; Ultra Centrifuge (BIO-LAB, BL-165D; India) and washed thrice with Sterile Triple Distilled Water (TDW) before transfer to fresh growth medium. The initial cell density was adjusted to 35µg protein ml^{-1} culture (absorbance 0.02; unless otherwise specified for a particular experiment) to assess the long term (2-8 days) impact of copper (as $CuSO_4.5H_2O$; Merk, Germany) on the growth rate / yield.

The algae were capable of forming homogenous suspension in liquid cultures and discrete pin-head colonies on nutrient agar. The Allen and Arnon's (1955) medium, free from any combined nitrogen sources (designated as AA-N), was used as the basal growth medium (pH 8.0) with A_6 trace elements devoid of copper. Agar-agar was added

PLATE : 2



(A)



(B)

Effects of Copper on *Nostoc calcicola* Breb
(A) Normal vegetative cell of *Nostoc calcicola* Breb
(B) *Nostoc calcicola* Breb showing cellular deleterious effects at 5µM lethal concentration of Cu.

(1% w/v) to obtain solid nutrient medium if desired. All such chemical ingredients in the medium were products either of Central Drug House Pvt. Ltd., New Delhi, or Qualikems Fine Chemicals Pvt. Ltd., New Delhi, India. Glass-wares used were of Borosilicate made. The culture medium, Glass-wares and Chemicals, were sterilized in an autoclave (Jyoti Scientific Industries, Gwalior, M.P., India), at 1.055 Kg cm^{-2} pressure for 15 min. Chemicals to be affected by such conditions were directly dissolved in sterile Triple Distilled Water (TDW) and filtered though Millipore filters (Porosity 0.45 µM, Schleischer and Schull, Germany). All the manipulations involving transfer of the organisms to fresh solid / liquid cultures were aseptically performed in a laminar flow hood (Ultra Scientific Industries, India).

4.2.1. ESTIMATION OF PROTEIN:-

Protein content of the cyanobacterial cultures was estimated by the method adopted by Lowry *et al.,* (1951), modified by Herbert *et al.,* (1971). The reagents employed were:-

- **Reagent "A":-** 1.0 N NaOH,
- **Reagent "B" :-** (a) 5.0 % Na_2CO_3,

(b) 0.5 % $CuSO_4.5H_2O$ in 1.0% Sodium potassium tartarate.

Chemicals (a) and (b) were mixed in the ratio of 25: 1 (v/v).

- **Reagent "C":-** 1.0 N Folin-phenol reagent.

PLATE : 3

(A) Mother Culture of *Nostoc calcicola* in Allen-Arnon Growth Medium under control Conditions

(B) Color of Protein developed after completing the experiment by following the method of Lowry *et al.*, 1951.

PROCEDURE:-

The estimation procedure was common to free as well as immobilized cells.

- Cells from beads were released by dissolving the alginate in 0.1M sodium citrate.
- A 0.5 ml of Reagent "A" was added to 0.5 ml algal culture and incubated for 5 min in a boiling water-bath.
- After sufficient cooling, 2.5 ml of Reagent "B" was added, and the reaction mixture incubated for 10 min at 37°C. This was followed by the addition of 0.5 ml Folin-phenol reagent and incubation for another 15 min.
- The intensity of the resulting blue color was determined at 650 nm, and the amount of algal cell protein calculated as µg ml^{-1} culture by reference to a standard calibrated curve, obtained with lysozyme (Sigma, USA).

measured

never used as protein standard.

should be calculated per biomass.

4.3. CELL IMMOBILIZATION:-

The method for cell immobilization is essentially same as done by Singh *et al.,* 1989.

- The cyanobacterial cells (400µg protein ml^{-1}) procured though centrifugation and repeated washings were suspended in 5% (w/v) solution of alginic acid prepared in the growth medium described before.
- The mixture was pumped out drop-wise into 0.2 M $CaCl_2$ solution aseptically in a laminar-flow cabinet.

➢ The beads thus formed, were subsequently harvested, resuspended in 200ml basal medium contained in cotton-stoppered Erlenmeyer flasks (capacity, 500 ml) and incubated photoautotrophically under culture room conditions along with the free cell sets.

4.4. COPPER UPTAKE KINETICS:-

4.4.1. COPPER UPTAKE EXPERIMENTS WITH FREE AND IMMOBILIZED CELLS OF *Nostoc calcicola*:-

➢ Log phase free cells of *Nostoc calcicola* were centrifuged, washed repeatedly with triple glass distilled water and suspended in the phosphate buffer (0.01M, pH 8.0) to a final density of 400µg protein ml culture. The metal uptake was observed at increasing Cu concentrations (10 to 60µM as $CuSO_4.5H_2O$). Similarly, Cu uptake was followed in immobilized cells.

not clear.

➢ Both the uptake experiments were conducted in light (14.4 Watt m^{-2}) at 24 ± 1°C.

➢ Samples were removed at desired intervals over 1 h and monitored for cellular Cu intake as well as adsorption. The intra-cellular Cu concentration was monitored by Atomic absorption spectrophotometer.

4.4.1.1. EDTA-WASHABLE METAL FRACTION (ADSORPTION):-

➢ The free cells (10 ml, after centrifugation) as well as beads recovered simultaneously at different time intervals of Cu exposures

were suspended in aqueous EDTA (10μM; disodium salt; BDH, UK) to account for the EDTA-washable metal fraction.

➢ Cu depleted from the uptake medium for both the sets, was estimated in a Perkin-Elmer, AAS-1450 atomic absorption spectrophotometer at 324.7nm. Cellular intake is expressed as the difference between Cu adsorption and the total amount of Cu depleted from the medium in which the free cells or beads remained suspended during Cu exposure.

4.4.2. ACTIVE AND PASSIVE COPPER UPTAKE:-

Free cells were toluenized to achieve permeabilization and subsequently immobilized in order to present the possible distinction between active and passive uptake of Cu.

4.5. FACTORS REGULATING COPPER UPTAKE:-

4.5.1. LIGHT AND EXOGENOUS ATP:-

Free cells and beads prepared from the same age free cell stock were dark-incubated for 72 h at 24 ±1°C. Since 10.0μM ATP was non-growth inhibitory to the organism in earlier report (Singh, C.B., 1987). The same concentration was applied to dark-incubated free and immobilized cells during the subsequent monitoring of Cu uptake.

4.5.2. INHIBITORS / UNCOUPLERS:-

DCCD (N, N'-dicyclohexycarbodiimide), pCMB (p-chloromercuribenzoate) were obtained from Sigma (USA) and 2-mercaptoethanol and NaN_3 from BDH, India. DCCD was solubilized in ethanol and added to the assay medium in a way that the final ethanol concentration never exceeded 0.1% (v/v; non-inhibitory to cell growth). Likewise, pCMB was dissolved in 0.1 N NaOH initially, and azide in sterile water. All such chemicals were added separately to the free and immobilized *Nostoc calcicola* containing a common Cu concentration (60μM Cu) for free and immobilized cells.

4.5.3. COPPER UPTAKE AND CULTURE AGE:-

Such experiments were aimed at comparing the metabolic longevity of free and immobilized cells. Cu uptake was monitored every 6^{th} day lasting 30 days in free as well as immobilized cells maintained initially at the same level of protein.

4.6. INTERACTION OF COPPER WITH PHOSPHATE UPTAKE, AND *ATPase*: -

4.6.1. PHOSPHATE UPTAKE:-

Inorganic phosphate present in the test medium was estimated following the method of Fiske and Subba Rao (1925).

PRINCIPLE:-

When inorganic phosphate reacts with molybdic acid, it forms phosphomolybdate. When ANSA (1-amino-2-nephthol-4-sulphonic acid) reagent is added, it reduces to blue colored compound that can be estimated at 660 nm.

REAGENTS: -

(a). 5 N- H_2SO_4

(b). 2.5% Ammonium-Molybdate $(NH_4)_2MoO_4$; 2.5gm dissolved in 100 ml water.

(c). Reducing agent prepared by mixing the following reagents in 100 ml water and stored in colored bottle:-

(i) 1.2 gm Sodium Metabisulphite,

(ii) 1.2 gm Sodium Sulphite,

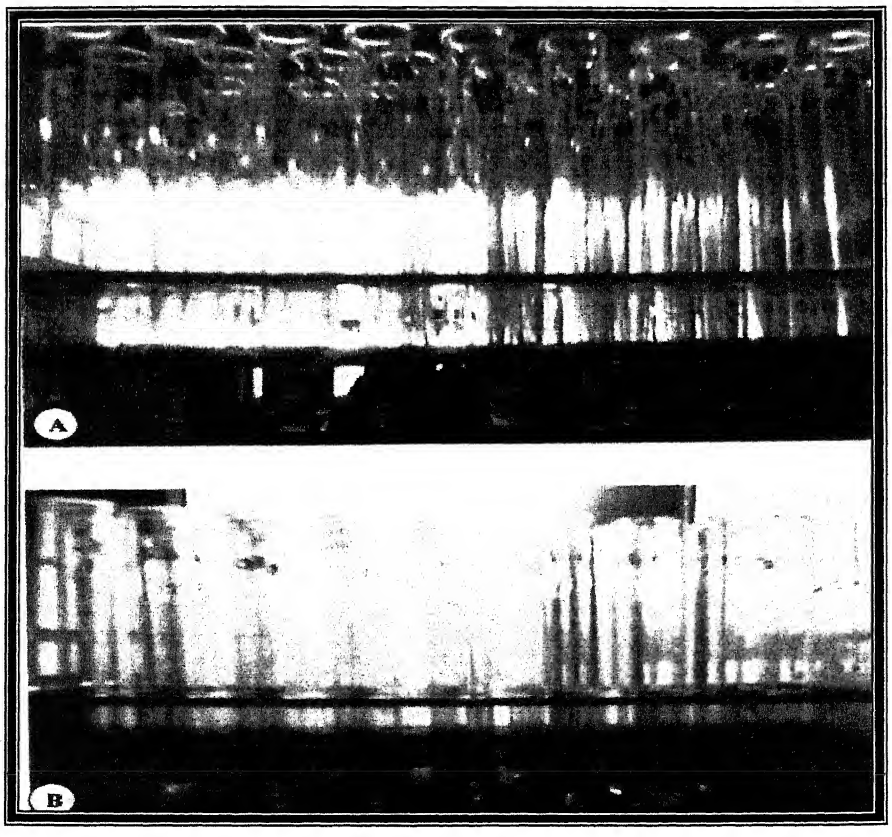
(iii) 0.2 gm ANSA (1-amino-2-nephthol-4-sulphonic acid),

(d). K_2HPO_4 Solution Standard.

PROCEDURE:-

- To 1 ml of standard (K_2HPO_4) solution or Culture supernatant, was added 1 ml 5 N-H_2SO_4, 1 ml ammonium molybdate and 0.1 ml Reducing agent (c), appropriate dilution achieved in water (final 10 ml volume), and the mixture incubated for 10 min. Optical density of the blue color so developed, was read at 660 nm.
- For Phosphate uptake experiments 6 days old free and immobilized *Nostoc calcicola* cells were starved for 12 h in a phosphate free medium.

PLATE : 4



(A). Color developed after completing the experiment **"Phosphate uptake"** by following the method of Fiske and Subba Row (1925).
(B). Experiment showing **Dilution *vs.* Cu-Efflux.**

- Experiments regarding cyanobacterial phosphate uptake proceeded as stated before using a wide range of K_2HPO_4 concentration (0.2-2.5mM for free cells and 0.5-3.0 mM for immobilized cells, respectively).
- As 2.0mM and 2.5mM K_2HPO_4 concentrations were saturating for free and immobilized cells, respectively; these were subsequently employed in experiments with different Cu concentrations (20µM to 80µM for free immobilized cells).

4.6.2. ESTIMATION OF *ATPase* ACTIVITY:-

4.6.2.1. ENZYME EXTRACTION:-

- *ATPase* activity of the cyanobacterial cells was assayed according to the method of Lockau and Pfeffer (1982) with little modification.
- The 6 days old free cells were harvested by centrifugation (5000x g); washed and re-suspended in extraction buffer (30 mM Tris-HCl, pH 8.1).
- A definite number of 6 days old beads having protein value equal to the free cells (66.4 mg protein) was taken, washed and re-suspended in extraction buffer (30 mM Tris-HCl buffer, pH 8.1).
- The cells from both the states were ruptured in liquid nitrogen followed by centrifugation at 4°C for 30 min (10,000x g).
- The supernatant thus obtained, was dialysed for 3 h against 10 mM Tris-HCl buffer (pH 8.1) and the resulting preparation was used as crude enzyme extract. All such operations were carried out at 4 C.

• Mg^{2+}-DEPENDENT *ATPase* :-

➢ Mg^{2+}-dependent *ATPase* was assayed by determining the amount of inorganic phosphate liberated as described by Ohnishi *et al* (1975). The assay mixture (2.0 ml) contained 6mM $MgCl_2$ and 6mM ATP (neutralized salt) in 30mM Tris-HCl buffer (pH 8.1).

➢ The reaction was initiated at 37°C by adding appropriate amount of the above enzyme preparation. The reaction was stopped at regular interval of 1 h by 0.25 ml Trichloroacetic acid (40%, Merk, Germany).

➢ The amount of inorganic phosphate liberated, was determined by the method of Subba Rao (1925).

• Ca^{2+}-DEPENDENT *ATPase* :-

➢ The Ca^{+2}-dependent *ATPase* enzyme preparation attributed to the coupling factor of photo-phosphorylation (Ower-Norhi *et al.,* 1979).

➢ The enzyme was activated prior to assay.

➢ The cell free extract was treated with *trypsin* (Sigma, USA; 0.5 mg ml^{-1}) for 10 min, followed by addition of 0.75 mg ml^{-1} of *trypsin* inhibitor (Sigma, USA).

➢ Ca^{+2}-dependent *ATPase* assay was performed as above except that $MgCl_2$ was replaced by 6mM $CaCl_2$ solution.

4.7. COPPER EFFECT ON THE *IN VIVO* ACTIVITY OF *NITROGENASE* AND *GLUTAMINE SYNTHETASE* (*TRANSFERASE*):-

4.7.1. COPPER EFFECT ON THE *IN VIVO* ACTIVITY OF *NITROGENASE*:-

The *in vivo nitrogenase* activity in *Nostoc calcicola* (free cells and beads) was measured by monitoring the amount of ethylene produced by the acetylene reduction technique (Stewart *et al.*, 1968).

- The 6 days old N_2-grown free as well as immobilized cyanobacterial cells adjusted to equal protein value (400µg protein ml^{-1}), were inoculated into fresh combined nitrogen-free growth medium containing graded concentrations of Cu (60 to 500µM for free and immobilized cells).
- A 2.0 ml volume of such metal dosed free cells and a definite number of beads (having equal protein value to that of 2.0 ml free cells) were taken into 8.5 ml capacity rubber-stoppered glass vials containing an atmosphere of 10% acetylene (sealed with parafilm).
- Such vials were continuously agitated in light and the reaction terminated at a regular interval of 30 min up to 3 h by injecting 0.2 ml 1.0 N HCl.
- The amount of ethylene in reaction vessels was determined in a gas chromatograph (Shimadzu, GC, Japan), fitted with Porapak R column. *Nitrogenase* activity is expressed as n mol C_2H_4 produced mg^{-1} protein min^{-1}.

4.7.2. COPPER EFFECT ON THE *IN VIVO* ACTIVITY OF *GLUTAMINE SYNTHETASE* (*TRANSFERASE*):-

The enzyme activity was calorimetrically monitored (Shapiro and Stadtman, 1970; Stacey *et al.*, 1977) by quantifying the amount of γ-glutamylhydroxamate produced in the following transfer reaction catalyzed by *glutamine synthetase*:

Divalent cation

$$\text{Glutamate} + NH_4^+ + \text{ATP} \longrightarrow \text{Glutamine} + \text{Pi} + \text{ADP}$$

glutamine synthetase

$$\text{Glutamine+Hydroxylamine} \longrightarrow \text{glutamylhydroxamate} + NH_3$$

ADP / Arsenate

One unit of the enzyme in this reaction has been defined as the amount of *glutamine synthetase* required to catalyze the synthesis of 1.0μ mol γ-glutamylhydroxamate min^{-1} under standard transfer assay conditions.

REAGENTS:-

(i). Extraction buffer (pH 7.0):-

- Imidazole HCl buffer (1.0 M; pH adjusted to 7.0 by HCl),
- L-glutamate (sodium salt, 1.0 M; pH 7-0) and
- $MgCl_2$ (1.67 M).

(ii). Reaction mixture (pH 7.0):-

- Glutamine (0.1M; pH 7.0),
- Potassium arsenate 1.0M (pH adjusted to 7.0 with KOH),
- Sodium ADP (0.01 M; pH adjusted to 7.0 with NaOH),
- Imidazole-HCl buffer (1.0 M; pH 7.0),
- $MnCl_2$ (0.1 M) and
- Hydroxylamine-HCl (2.0 M).

(iii). Stop mixture:-

- 4.0ml 10.0% FeCl, was mixed with 1.0 ml 24.0% trichloroacetic acid,
- 0.5ml 6.0 N HCl and
- 6.5ml distilled water.

PROCEDURE:-

• Dissociation of algal beads:-

As described before, the immobilized cells were released from alginate beads by dissolving the alginate in 0.1 M sodium citrate prior to centrifugation.

• *Glutamine Synthetase* (*transferase*) Activity :-

- The 6days old N_2-grown free *Nostoc calcicola* cells were inoculated into fresh growth medium (400µg protein ml^{-1} culture) containing

graded concentrations of Cu (60 to 500μM) and 1.0 ml algal sample taken out at 30 min interval for centrifugation.

- Similarly, 6 days old immobilized cyanobacterial cells (equivalent to 400μg protein) were also inoculated into fresh growth medium containing graded Cu concentrations (60 to 500μM).
- The pellets obtained by centrifugation of free as well as immobilized cells were subjected to toluenization in 0.5 ml toluene, concurrent rigorous shaking and incubation for 10 min at 4°C.
- The test tubes were shaken again to ensure complete permeabilization of cell membrane followed by centrifugation to discard the toluene top layer.
- The cell extract was dissolved in 0.5 ml extraction buffer and treated with 0.8 ml reaction mixture.
- The reaction was terminated after 30 min incubation at 37°C by adding 2.0 ml stop mixture.
- The turbid debris in the resultant solution was removed by centrifugation. The intensity of the coffee-color solution read at 540 nm against the reagent blank, prepared by eliminating glutamine and hydroxylamine from the reaction mixture.
- *Glutamine synthetase* (*transferase*) activity is expressed as μ mol γ-glutamylhydroxamate produced mg^{-1} protein min, as quantified by reference to a standard curve obtained with γ-glutamylhydroxamate (Sigma, USA) in the assay mixture.

4.8. $^{14}CO_2$-INCORPORATION:-

- Photosynthesis in *Nostoc calcicola* was measured as a function of $^{14}C0_2$-fixation from $NaH^{14}CO_3$ (0.05μCi/ml) obtained from the Isotope Division, BARC, India.
- The photoautotrophically grown 6 days old free and immobilized cyanobacterial cells after transfer to fresh growth medium, were pre-incubated in dark 24 ± 1°C) for 24 h.
- A 1.0 ml volume of the dark incubated cyanobacterial free cells (400μg protein ml^{-1} culture) and beads (corresponding to the same protein value), were transferred to glass scintillation vials (Beckman, USA), containing graded Cu concentrations (30μM & 60μM for free and immobilized cells) and 0.05μCi ml^{-1} $NH^{14}CO_3$.
- The simultaneously run metal-less control and metal-treated free and immobilized cells in the scintillation vials, were light-incubated at 24 ± 1°C and $^{14}CO_2$-fixation stopped at regular intervals of 1 h by adding 0.1 ml 2.0 N HCl, followed by the addition of 5.0 ml scintillation cocktail, containing 4 parts of 0.8% PPO (2,5-diphenyloxazole; Sigma, USA) plus 0.01% POPOP (1,4-bis (4-methyl-5-phenyl-2 oxazole)-benzene; SIGMA, USA) in toluene and 3 parts of ethanol.
- Such reaction mixtures were surface blown for 5 min to remove the $^{14}CO_2$ gas, and the clear solution subjected to counting the emission of β-particles from incorporated $^{14}CO_2$ in a liquid scintillation counter (Beckman, Model LS-7000, USA). The value of counts obtained is expressed as CPM mg^{-1} protein.

4.9. STUDIES ON COPPER RESISTANT / TOLERANT STRAIN OF *Nostoc calcicola*:-

4.9.1. ISOLATION OF COPPER-RESISTANT (Cu^r) STRAIN OF *Nostoc calcicola*:-

Log phase population of present algae *Nostoc calcicola* Breb with a cell density of approximately $1x10^5$ cells per ml was seeded on nutrient agar prepared in nitrogen free Allen and Arnon medium (1955) containing different copper levels (0-100μM) and subsequently shifted to growth conditions in culture room as mentioned earlier. As cyanobacterial cells could not grow in 60μM Cu, this form the basis of isolation of colonies, growing in the presence of 70μM Cu under diazotrophic condition. The general methodology for scoring the copper resistant strain was essentially the same as described by Singh and Singh (1978). The Cu^r strain clones grown as nutrient plates arose with a frequency of $2 x10^{-7}$, as calculated by the method of Stewart and Singh (1975) and all of these were adopted for further studies. Its stability was checked though replating on nutrient plates containing 70μm Cu. This population was grown in bulk.

4.9.2. COPPER EFFLUX EXPERIMENTS:-

➢ Exponentially growing *Nostoc calcicola* cells (Cu-sensitive as well as Cu-resistant) were centrifuged and washed twice with sterile

TDW and suspended in phosphate buffer (0.01 M; pH 8.0) to a final density of 400µg protein ml^{-1}.

- Such cells were incubated in buffer containing 60µM Cu.
- Experiment was conducted for 1 h followed by centrifugation and repeated washing with 10µM EDTA to remove absorbed Cu.
- The pellets of both (Cu^s and Cu^r) sets were re-suspended in Cu-free phosphate buffer.
- The metal amount liberated from such cells in buffer accounted for Cu extruded from either Cu^s or Cu^r strains.
- Cu efflux was monitored as desired intervals (0-60 min) and expressed as n mol Cu extruded mg^{-1} protein.

4.9.3. FACTORS REGULATING COPPER EFFLUX: -

4.9.3.1. LIGHT AND EXOGENOUS ATP:-

- Log phase cells of Cu^r strain were pre-exposed to 60µM Cu for 1 h and incubated in dark at 24 ±1°C during Cu efflux experiments.
- Since 10µM ATP was non-inhibitory to cyanobacterial growth, it has been used for Cu efflux experiments (Verma and Singh, 1990).
- Same ATP concentration was applied to dark incubated as well as photoautotrophically grown cells.

4.9.3.2. DILUTION *VS.* COPPER-EFFLUX :-

Cu^r cells were centrifuged and exposed to 60μM Cu for 1 h followed by repeated washing with 10μM EDTA.

- Such cells were incubated in basal medium having different Cu-dilutions 10, 100, 250, 500, 750 and 1000-fold corresponding to final Cu-concentration of 6, 0.6, 0.24, 0.12, 0.08 and 0.06μM respectively.
- Cu efflux was monitored during 0-60 min as described before.

4.9.3.3. INHIBITORS / UNCOUPLERS:-

DCCD (N, N'-dicyclohexycarbodiimide), pCMB (p-chloromercuribenzoate) were obtained from Sigma (USA) and 2-mercaptoethanol and NaN_3 from BDH, India. DCCD was solubilized in ethanol and added to the assay medium in a way that the final ethanol concentration never exceeded 0.1% (v/v; non-inhibitory to cell growth). Likewise, pCMB was dissolved in 0.1 N NaOH initially, and azide in sterile water. All such chemicals were added to the Cu^r strain that regulate Cu-efflux by different rates.

4.9.3.4. INTRACELLULAR COPPER *VS.* COPPER EFFLUX:-

- Exponential Cu^r cells were centrifuged and washed twice with Sterile TDW and re-suspended in basal medium containing 60μM Cu.
- A 10 ml aliquot was taken at regular intervals over 1 h, centrifuged and re- suspended in Cu-free medium for 1 h.
- Cu efflux was monitored in such cells at 1 h as described before.

CHAPTER – 5.

EXPERIMENTAL RESULTS

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EXPERIMENTAL RESULTS

5.1. COPPER UPTAKE:-

5.1.1. COPPER UPTAKE KINETICS:-

Free and immobilized cells were tried for their Cu-uptake response with respect to different increasing Cu concentrations. Free cells (initial cell density 400µg protein ml^{-1}) took up Cu from the ambient medium in a concentration-dependent manner (10 to 60µM). The intracellular buildup is 96.89 n mol Cu mg^{-1} protein for free *Nostoc calcicola* cells. **(Figure:1).**

The immobilized cells (with the same initial population size), also showed a concentration-dependent Cu uptake with the intracellular buildup is 300.82 n mol Cu mg^{-1} protein for immobilized *Nostoc calcicola.* **(Figure:2).**

Therefore, 60µM Cu has been used as the Cu concentration in subsequent metal uptake experiment. A comparison in this regard showed that immobilized cells have more cellular Cu buildup.

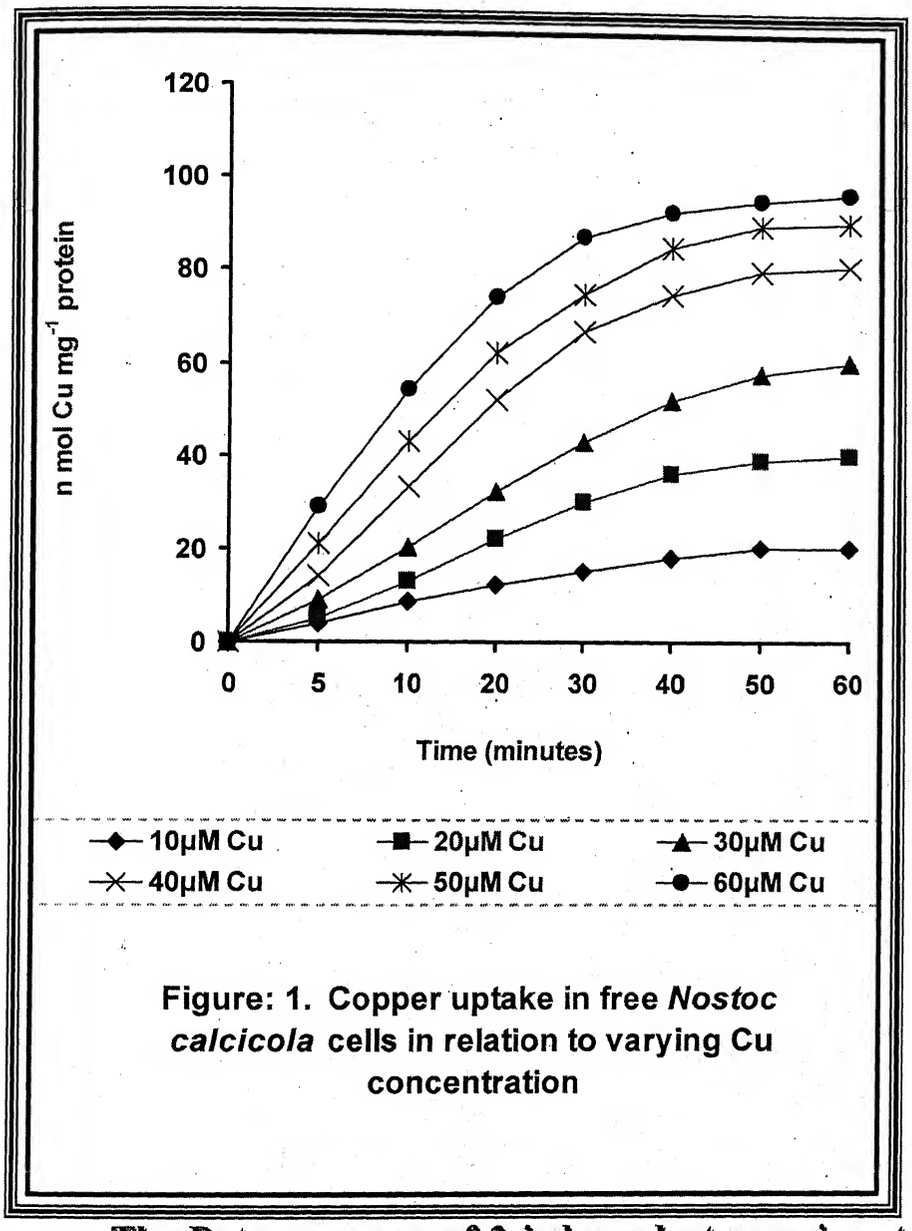

Figure: 1. Copper uptake in free *Nostoc calcicola* cells in relation to varying Cu concentration

The Data are mean of 2 independent experiments with 4 replicates of each. The maximum variation from the mean value was less than 5%. (Applicable for all the figures).

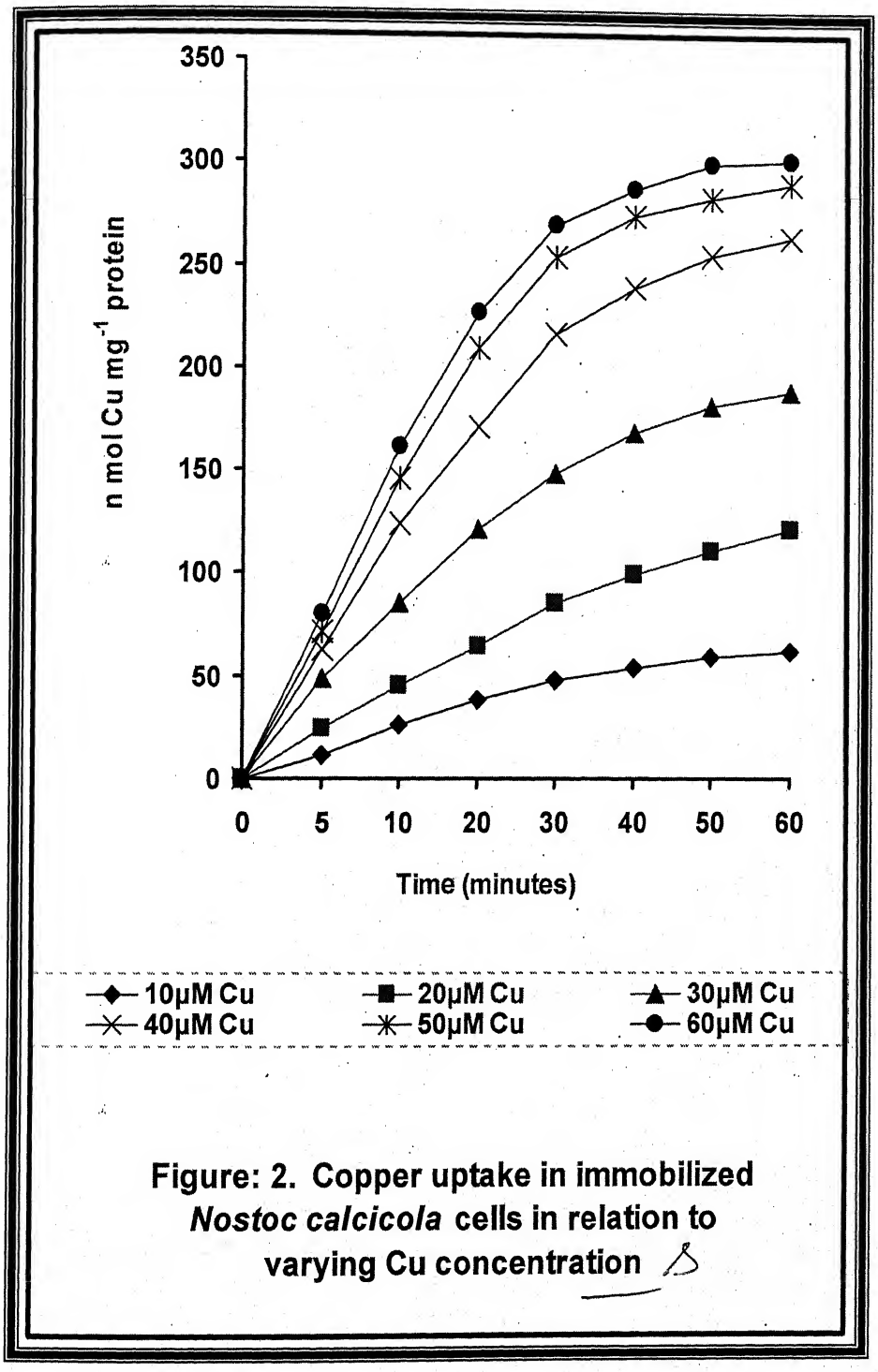

Figure: 2. Copper uptake in immobilized *Nostoc calcicola* cells in relation to varying Cu concentration

5.1.2. ACTIVE AND PASSIVE COPPER UPTAKE IN FREE AND IMMOBILIZED CELLS OF *Nostoc calcicola*:-

Experiments were conducted to compare the trend of energy-dependent Cu-uptake in free and immobilized cells. Separate experiments were also performed taking the toluenized free cells as well as immobilized cells in order to ascertain the active and passive uptake of Cu. Immobilized (toluenized) cells were obtained after immediate preparation of beads comprising of toluenized free cells. Toluenization of cyanobacterial cells were done keeping in view that as such cells gave the access to total diffusion independent of metal-carriers. Active uptake is light-dependent and carrier-mediated while the passive uptake (mainly diffusion) as the energy-independent event. **Figure: 3** represents a comparison of Cu-uptake by non-toluenized and toluenized free cells (maintained at the common cell concentration of 400μg protein ml^{-1}) up to 1 h in the uptake medium containing 60μM Cu. The pattern of curves was characterized by a steady rise in the Cu-uptake rate for non-toluenized (44.36 n mol Cu mg^{-1} protein min^{-1}) up to 10 min compared to toluenized cells (75.11 n mol Cu mg^{-1} protein min^{-1}) extending up to 10 min. The non-toluenized cells, however, showed a gradual decline in Cu-uptake rate beyond 10 min and a saturation initiating even at 50 min. The upper curve for Cu diffusion by toluenized cells also showed a gradual decline in the metal intake rate beyond the initial 10 min with the only difference that the process attained saturation beyond 20 min. Presuming that toluenization of free cells would permit passive diffusion of Cu, the difference of the total metal intake values in this case comes to 53.33 n mol Cu mg^{-1} protein

as the passive uptake while active uptake value (based on the lower curve) was still higher (96.89 n mol Cu mg^{-1} protein).

Likewise, **Figure: 4** represents an almost similar pattern of Cu intake in normal (untoluenized) cells, immobilized and those immobilized following toluenization. However, the Cu uptake rates for both these sets were invariably higher than that of free cells (refer **Figure: 3).** This is quite evident as the normal, immobilized cells maintained a rate 10 times that of free cyanobacterial cells (up to 60 min). On the assumption that the apparent higher Cu-uptake rate in immobilized cyanobacterial cells, following toluenization, was attributable to the passive diffusion of Cu (upper curve), the difference with those of non-toluenized cells (191.65 n mol Cu mg^{-1} protein) could be taken as the extent of passive uptake and 300.82 n mol Cu mg^{-1} protein as active uptake.

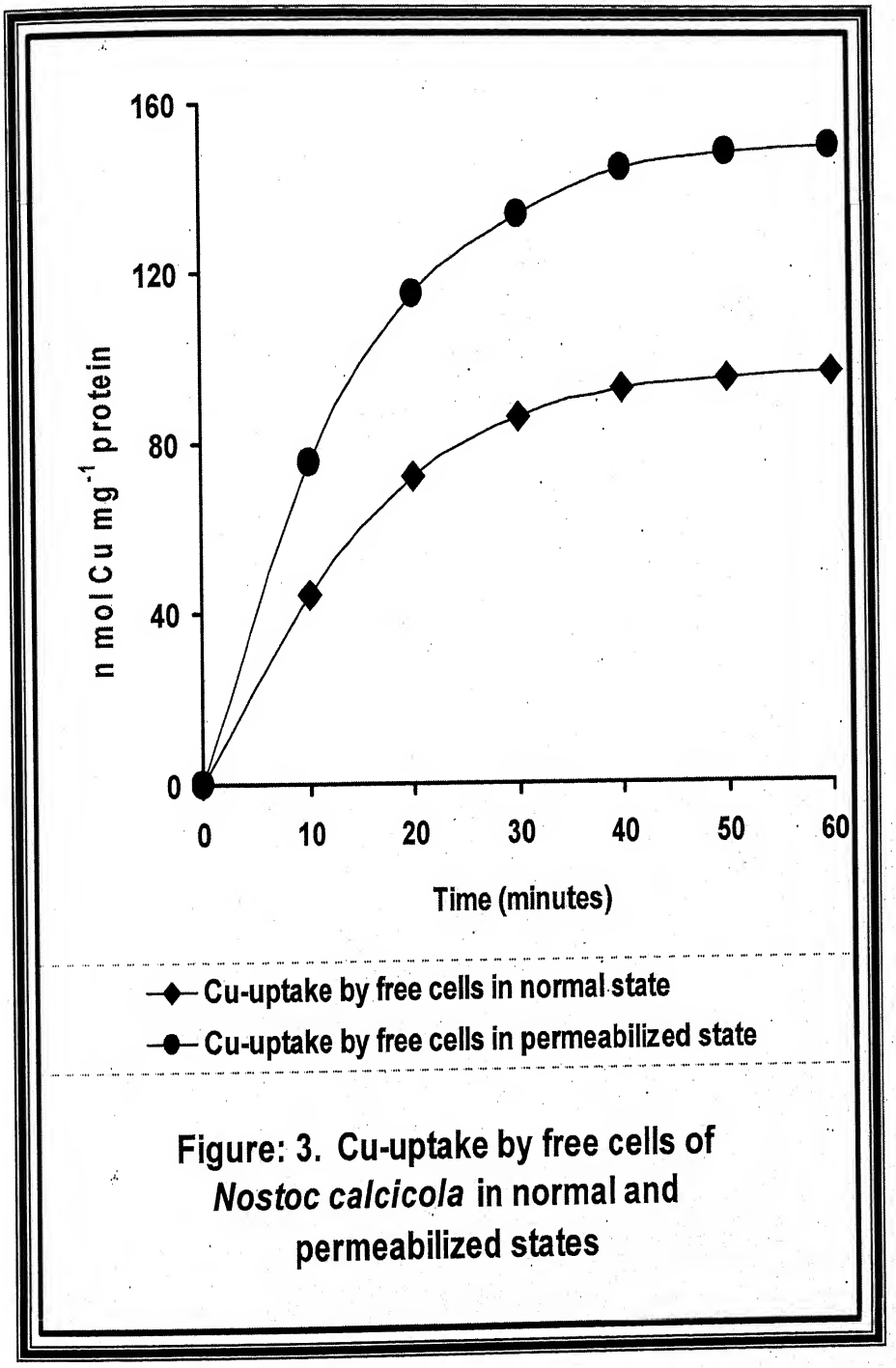

Figure: 3. Cu-uptake by free cells of *Nostoc calcicola* in normal and permeabilized states

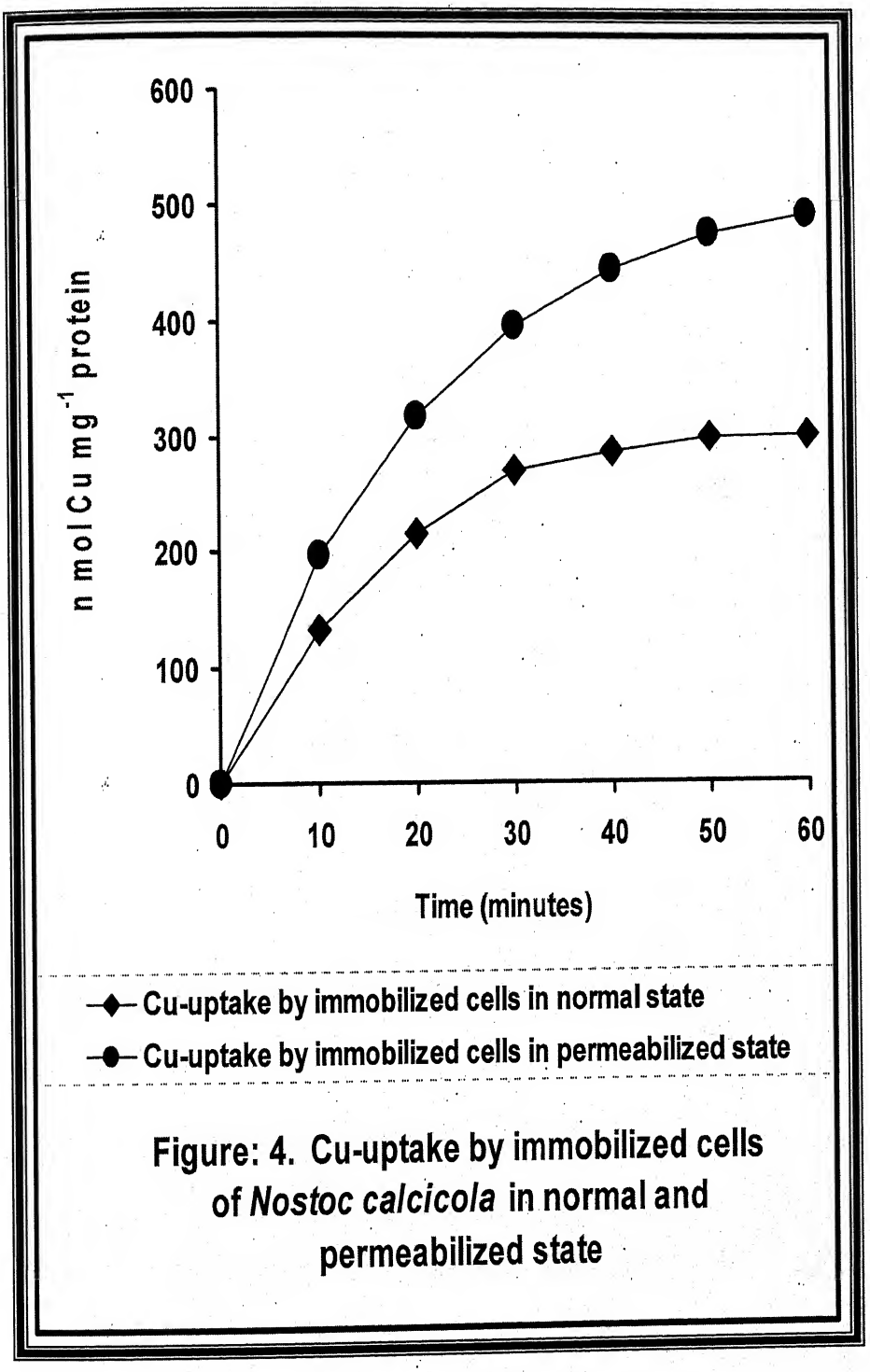

Figure: 4. Cu-uptake by immobilized cells of *Nostoc calcicola* in normal and permeabilized state

5.2. FACTORS REGULATING COPPER UPTAKE:-

5.2.1. EXOGENOUS ATP *VS.* COPPER UPTAKE:-

All the previous experiments involved metal uptake in light-grown cyanobacterial cultures and since such photoautotroph derive most of their cellular energy though photosynthesis, it was of interest to look for the possible role of exogenously added ATP in metal uptake for dark-incubated cells. Dark-incubated means that the culture was incubated in dark for 72 h. Free cells exposed to dark (72 h) showed an abrupt decline in Cu uptake (7.16 n mol Cu mg^{-1} protein) amounting to 14-fold difference from the light-grown control cells (96.89 n mol Cu mg^{-1} protein). Such "dark" cells, when supplied with exogenous ATP (10μM), enhanced the level of Cu uptake by 10-fold (72.86 n mol Cu mg^{-1} protein). The incomplete recovery in Cu intake in such a case not reaching the "light" cells could be attributed to the short supply of other factor (reductants) in "dark" cells **(Figure: 5).** Immobilized cells, on the other hand, facing dark incubation reflected only a 33% lowering in Cu uptake (200.15 n mol Cu mg^{-1} protein) compared to light-grown beads (300.82 n mol Cu mg^{-1} protein). Such comparisons clearly indicate that immobilized cells have sufficient energy in reserve even if subjected to non-photosynthetic conditions **(Figure: 6).** A negligible recovery in "dark" beads supplemented with 10μM ATP (5%) compared to free cells just described, indicates the maintenance of sufficient stored ATP pool even if exposed to dark-stress (72 h). In this case also, the exogenously supplied ATP to dark beads, could not raise the Cu uptake to the level of "light" beads, thus, once again emphasizing the regulatory role of other factor(s), may be the reductant.

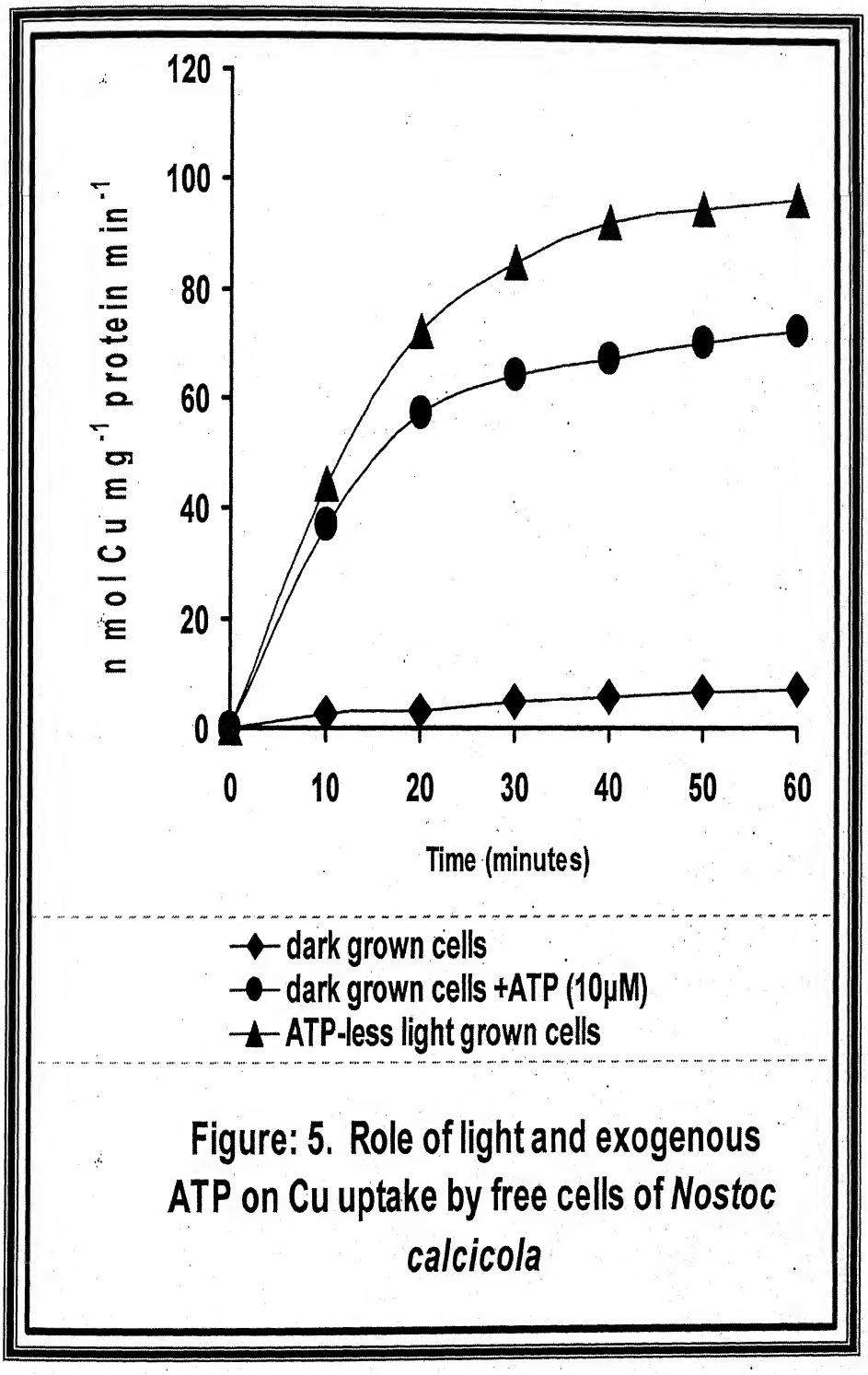

Figure: 5. Role of light and exogenous ATP on Cu uptake by free cells of *Nostoc calcicola*

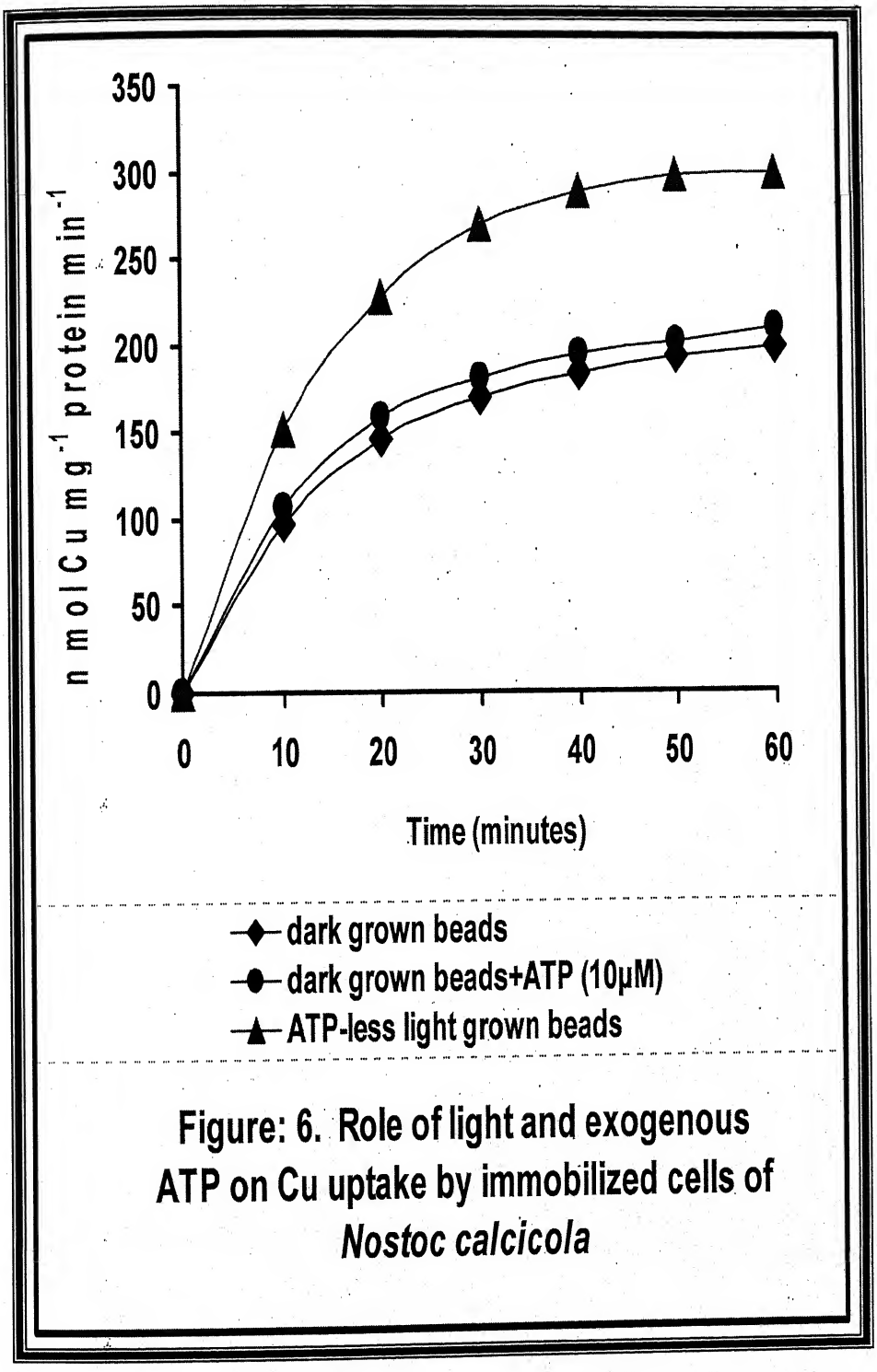

Figure: 6. Role of light and exogenous ATP on Cu uptake by immobilized cells of *Nostoc calcicola*

COMPARISON OF COPPER UPTAKE BY FREE AND IMMOBILIZED CELLS OF *Nostoc calcicola* AT A COMMON CONCENTRATION (60μM):-

Free cells reflected a relatively faster metal uptake rate for the first five min (6.0 n mol Cu mg^{-1} protein min^{-1}). However, such a pace could not be maintained any longer as it gradually decreased with the passage of time reaching a maximum 1.61 n mol Cu mg^{-1} protein min^{-1} (3.7-fold) at 60 min of metal exposure. A negligible difference in Cu-uptake values at 50 min (95.36 n mol Cu mg^{-1} protein) or at 60 min (96.89 n mol Cu mg^{-1} protein), also suggests that the maximum ~~The~~ intracellular Cu buildup is 96.89 n mol Cu mg^{-1} protein for free *Nostoc calcicola* cells. Cu-uptake by immobilized cells was characterized by an initial shoot up in rate to 16.24 n mol Cu mg^{-1} protein min^{-1}, thus corresponding to more than 2.5-fold increase over their free cell counterpart at 10 min **(Figure:7).** A close proximity of values at 50 min (299.97 n mol Cu mg^{-1} protein) and at 60 min (300.82 n mol Cu mg^{-1} protein) suggests that for immobilized cells also, the Cu buildup could have initiated prior to 1 h. The immobilized cells invariably maintained a higher profile of Cu intake amounting to 3.16-fold difference (300.82 n mol Cu mg^{-1} protein) over cells in suspension (96.89 n mol Cu mg protein). Such a difference could also be attributed to the correspondingly higher Cu-uptake rates (16.24 n mol Cu mg^{-1} protein min^{-1}) of immobilized cells than those of free cells (6.0 n mol Cu mg^{-1} protein min^{-1}).The corresponding rates of Cu adsorption by free (0.646 n mol Cu mg^{-1} protein min^{-1}; at 60 min) and immobilized (1.26 n mol Cu mg^{-1} protein min^{-1}; at 60 min) cyanobacterium indicate the greater efficiency of the latter.

In complete sentence

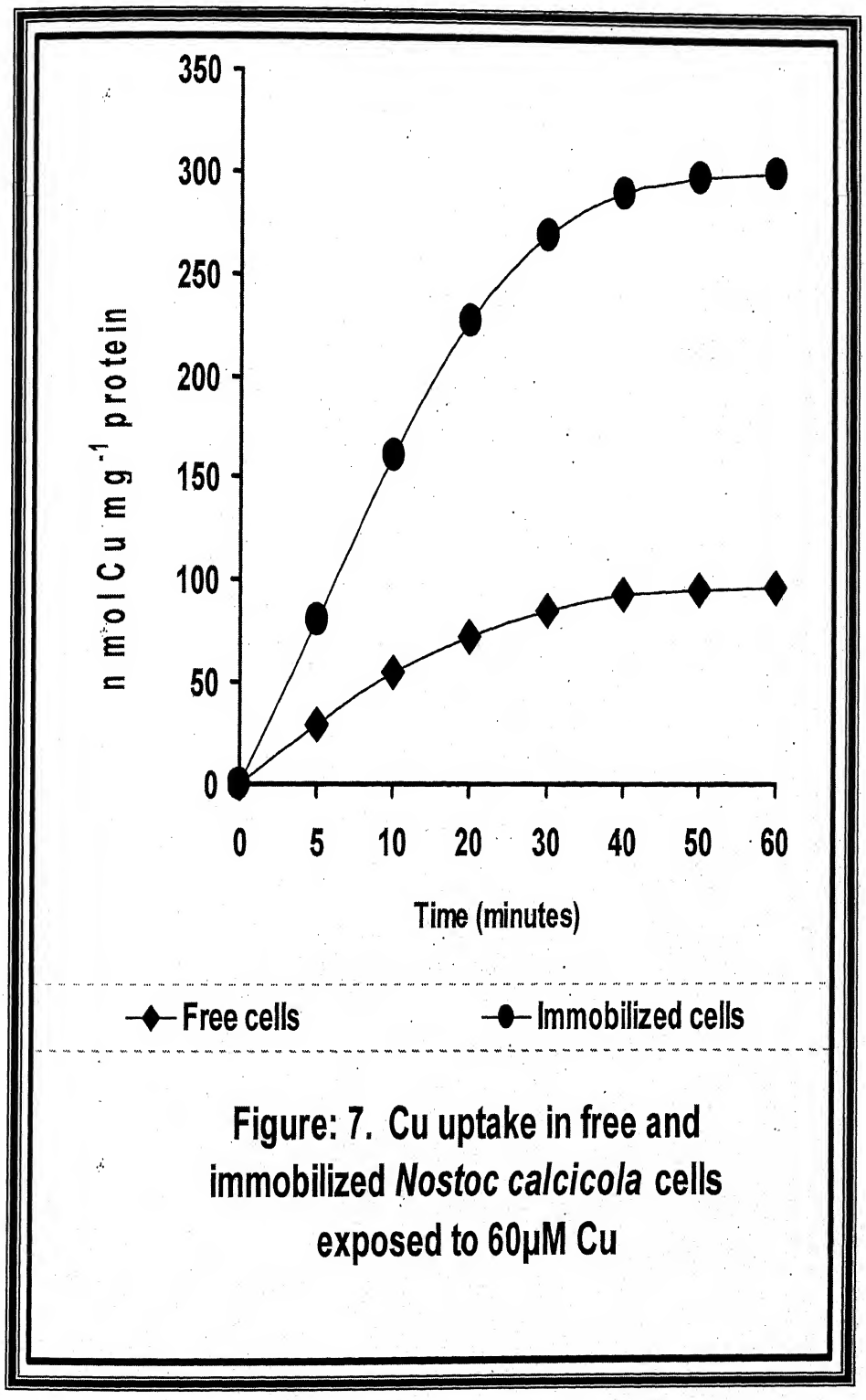

Figure: 7. Cu uptake in free and immobilized *Nostoc calcicola* cells exposed to 60µM Cu

5.2.2. METABOLIC INHIBITORS / UNCOUPLERS:-

The relative contribution of different metabolic inhibitors / uncouplers in regulating Cu-uptake in free cells of *Nostoc calcicola* is shown in **Figure: 8.** The Cu-uptake was inhibited by mercaptoethanol which reduced Cu-uptake by 25% (73.98 n mol Cu mg^{-1} protein). Sodium azide (10μM) inhibits respiratory electron transport and uncouples oxidative phosphorylation; caused approximately 50% inhibition of Cu-uptake. Cu-uptake was also influenced by DCCD which inhibited 75% Cu-transport. On the other hand Cu-uptake in free cells completely inhibited by pCMB (1.0μM). **Figure:9** shows the role of various metabolic inhibitors / uncouplers on Cu-uptake in immobilized *Nostoc calcicola* cells, more or less similar results were obtained.

5.2.3. CELL AGE *VS.* COPPER UPTAKE:-

As the cell age is also an indication of the metabolic state, it was of interest to compare Cu-uptake in free and immobilized cells simultaneously within a time regime of 6 to 30 days as shown in **Figure: 10.** As the experimental protocol, two parallel sets contained (a) one with free cells inoculated into fresh growth medium, and (b) the same cell mass was converted into beads, and allowed to grow in liquid medium. Cu uptake was monitored in both the sets at 6 days interval.

The lower curve displays almost the same extent of Cu uptake (96.89 n mol Cu mg^{-1} protein) in free cells lasting 12 days, and invariably declined further with increase in the culture age.

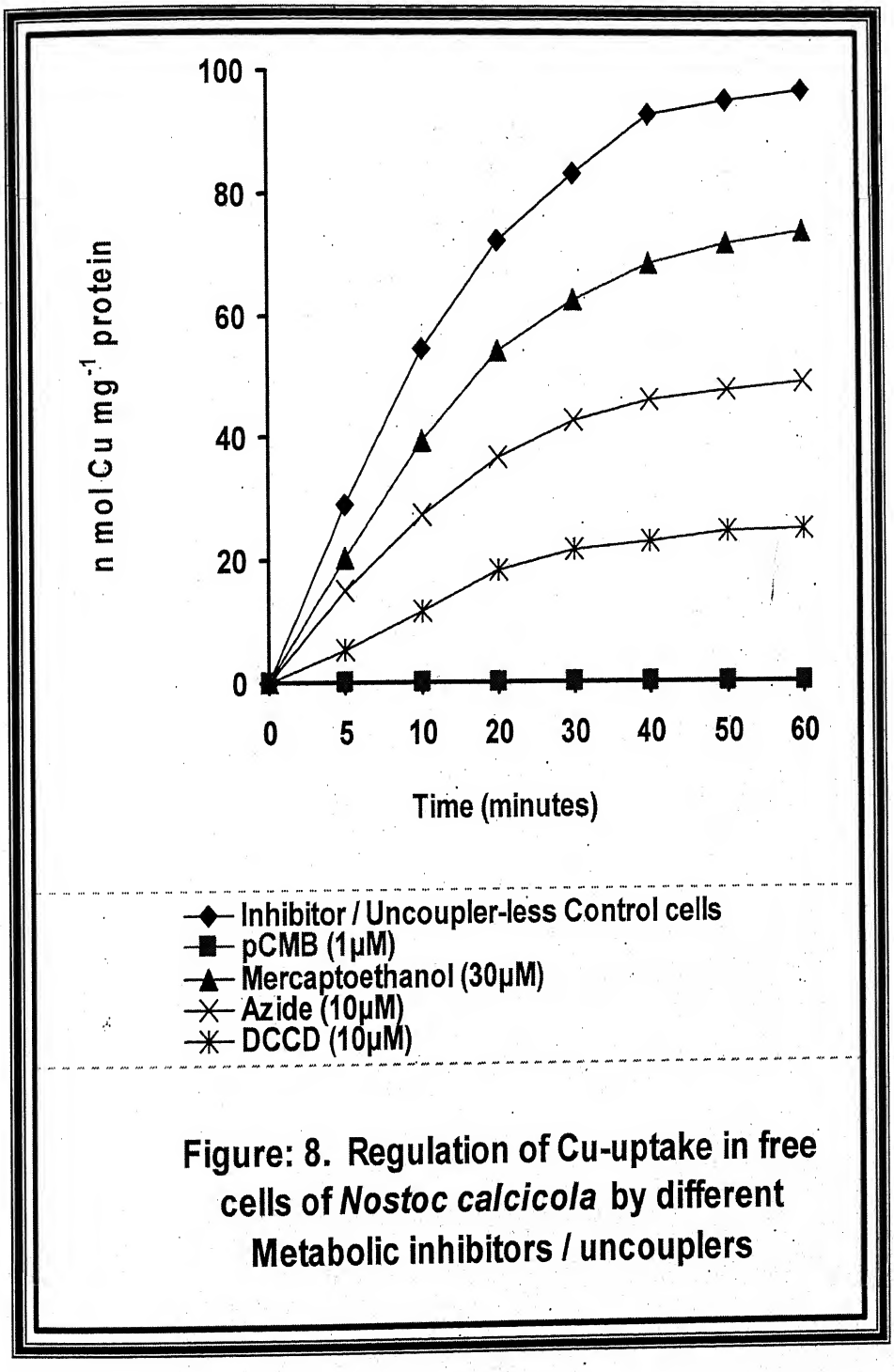

Figure: 8. Regulation of Cu-uptake in free cells of *Nostoc calcicola* by different Metabolic inhibitors / uncouplers

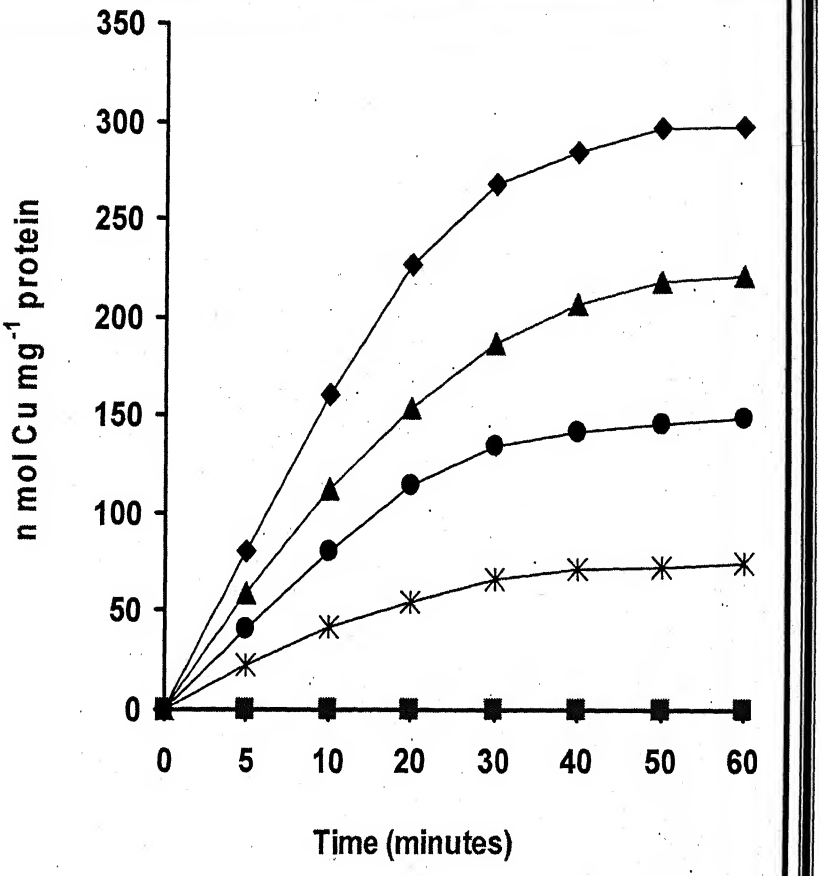

Figure: 9. Regulation of Cu-uptake in immobilized *Nostoc calcicola* cells by different Metabolic inhibitors / uncouplers

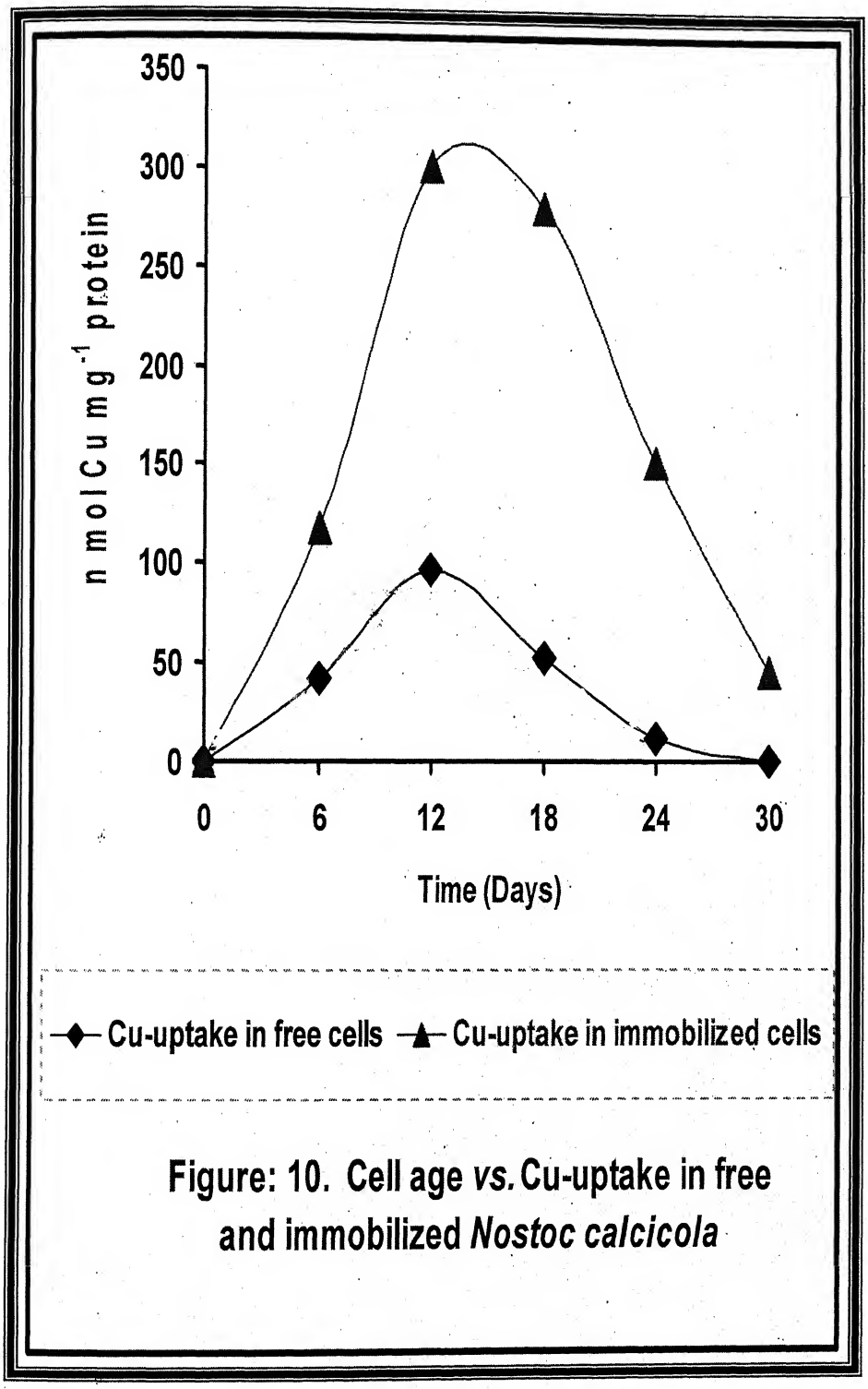

Figure: 10. Cell age *vs.* Cu-uptake in free and immobilized *Nostoc calcicola*

The extent of Cu-uptake for 6 days old beads is typical of their behaviour as observed earlier (3 times higher value of metal intake over free cells) of same age. Cells in this state seem to have prolonged their longevity as evident from the peak value of Cu intake (300.82 n mol Cu mg^{-1} protein) on12^{th} day.

5.3. INTERACTION OF COPPER WITH PHOSPHATE UPTAKE AND *ATPase*:-

5.3.1. INTERACTION OF COPPER WITH PHOSPHATE UPTAKE:-

At a stage, the cyanobacterial response to Cu toxicity or uptake had established that the metal could have inhibited general growth, it was of interest to look into the phosphorus transport in free and immobilized cells in response to Cu. Phosphorus uptake experiments were done in terms of PO_4^{3-} uptake kinetics in metal-less control cells and those exposed to varying Cu concentrations as applicable to free and immobilized cells. Cells / beads were pre-incubated in a PO_4^{3-} (phosphorus)-free growth medium for 12 h in light. Such cells responded rapidly to PO_4^{3-} supply without any lag.

Free cells **(Figure: 11)** took up PO_4^{3-} in almost a linear pattern up to 3 h with an indication of the trend towards saturation at 5 h (0.78μ mol PO_4^{3-} μg^{-1} protein). The overall pattern of Cu response of PO_4^{3-} uptake reflected a concentration as well as time-dependent inhibition with 50% reduction at 20μM Cu. The highest Cu concentration used in present case (80μM) when applied to PO_4^{3-} uptake test, brought about 91% inhibition of PO_4^{3-} uptake.

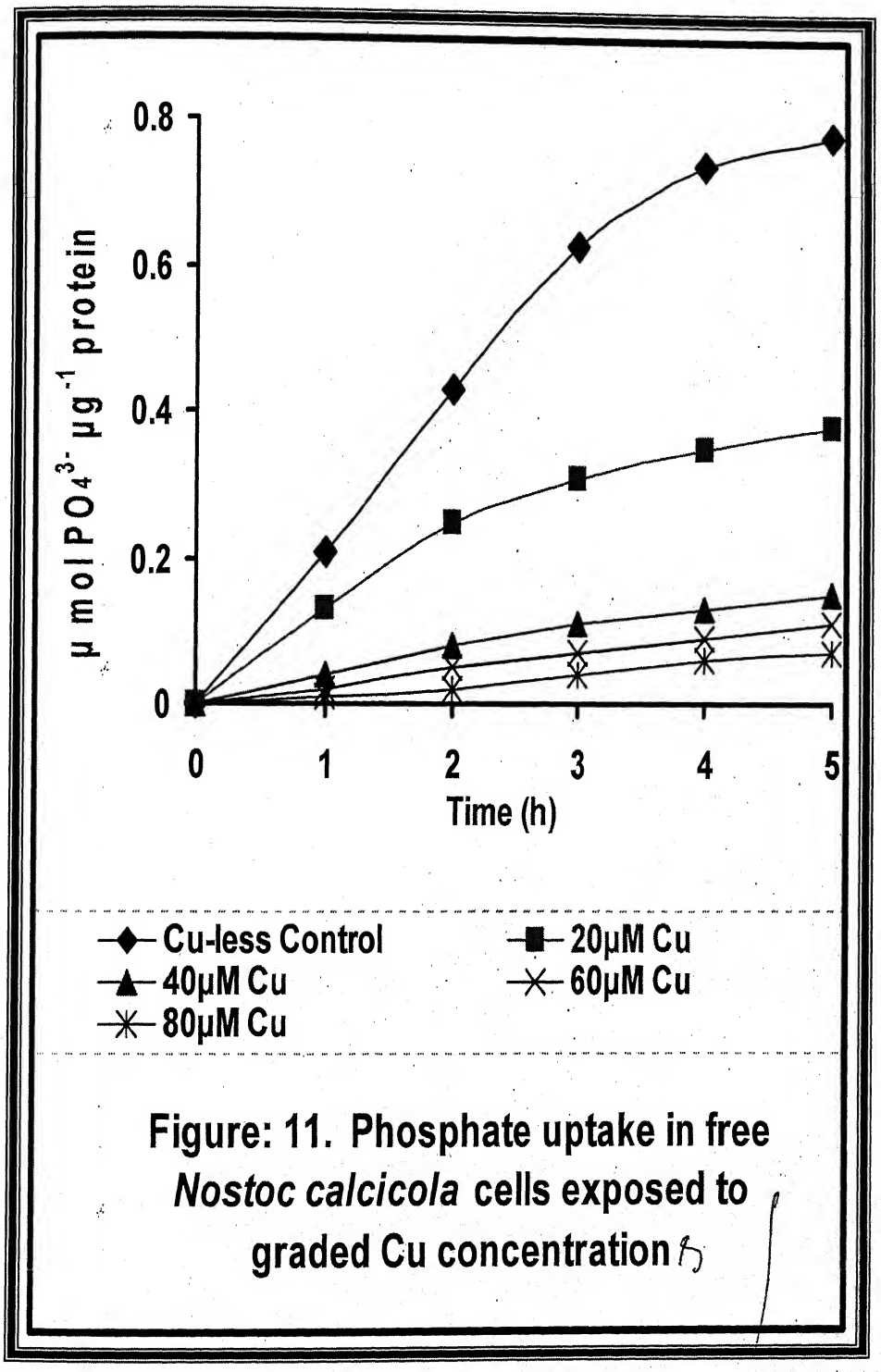

Figure: 11. Phosphate uptake in free *Nostoc calcicola* cells exposed to graded Cu concentration

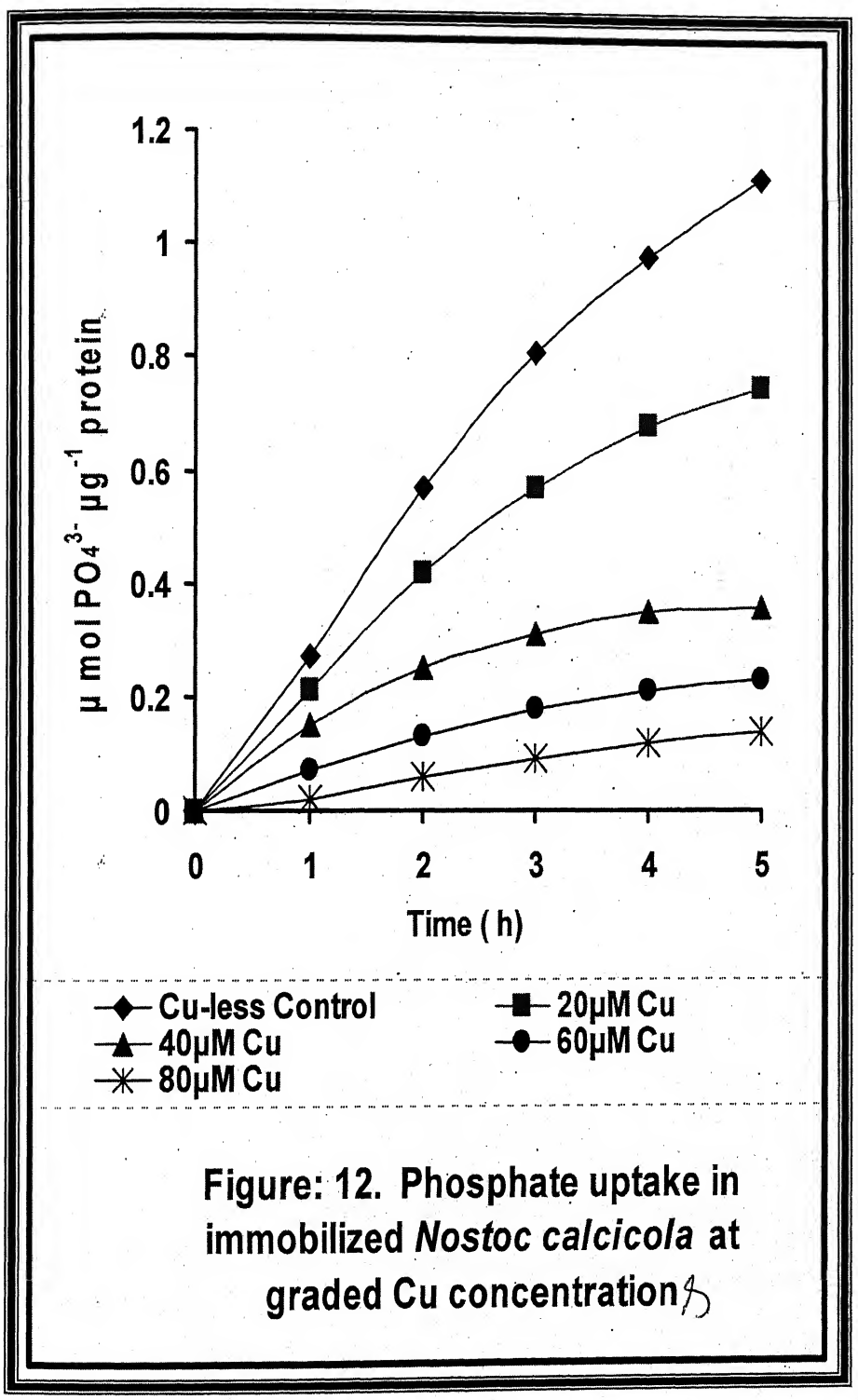

Figure: 12. Phosphate uptake in immobilized *Nostoc calcicola* at graded Cu concentration

The immobilized cells like the previous comparisons were also characterized by a faster rate of PO_4^{3-} intake (0.22μ mol PO_4^{3-} μg^{-1} protein h^{-1}; comparison of **Figures: 11 & 12).** Cells in immobilized state also responded to increasing Cu concentrations, although sufficiently high Cu amount (80μM) reflected less than 80% inhibition in PO_4^{3-} uptake.

5.3.2. INTERACTION OF COPPER WITH *ATPase*:-

5.3.2.1 *ATPase* ACTIVITY:-

The effect of Cu on Mg^{2+}-dependent *ATPase* activity (*in vivo*) in free cells of *Nostoc calcicola* is shown in **Figure: 13.** Cu-less control cells showed a basic level of 82.52 n mol Pi liberated mg^{-1} protein min^{-1} at 3 h. 40μM Cu-treated cells on the other hand showed a slightly higher Mg^{2+}-dependent *ATPase* activity (*in vivo*) at 1 h **(Figure: 13).** It also showed a declining pattern with a 30% activity remained at 3 h whereas the highest used Cu concentration (80μM) showed minimum Mg^{2+}-dependent *ATPase* (*in vivo*) activity (76.17 and 19.79 n mol Pi liberated mg^{-1} protein min^{-1} at 1 h and 3 h respectively) in free cells. However, the highest Cu-concentration (80μM) showed 75% inhibition in enzyme activity (19.79 n mol Pi liberated mg^{-1} protein min^{-1}) in free cells.

Figure: 14 reflect the data on the effect of Cu on Mg^{2+}-dependent *ATPase* activity (*in vivo*) in immobilized cells. The data showed that immobilized cells had always a lower profile as compared to free cells counterpart. The inhibition pattern was almost similar as in case of free cells.

not applicable at 1h duration

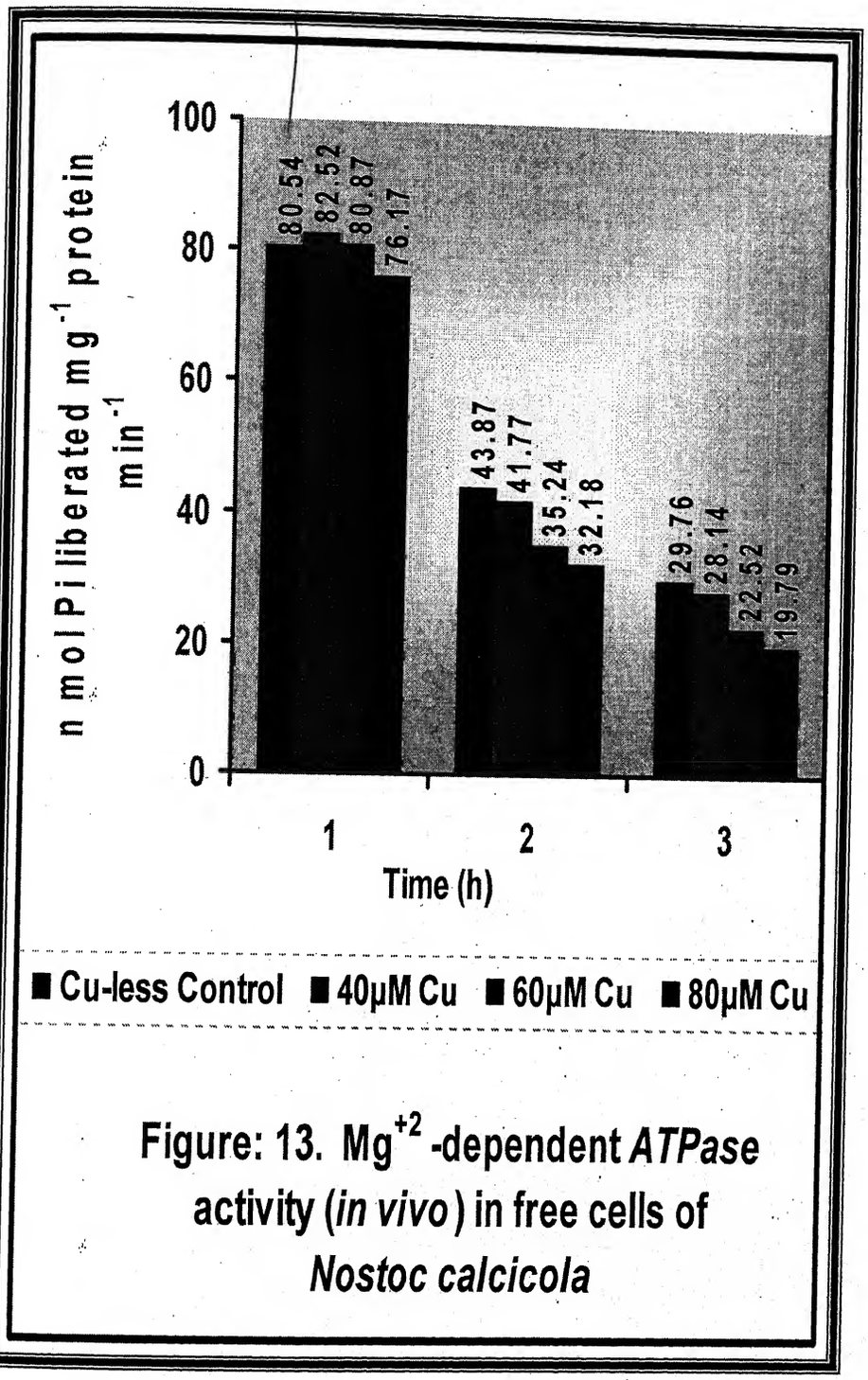

Figure: 13. Mg^{+2} -dependent *ATPase* activity (*in vivo*) in free cells of *Nostoc calcicola*

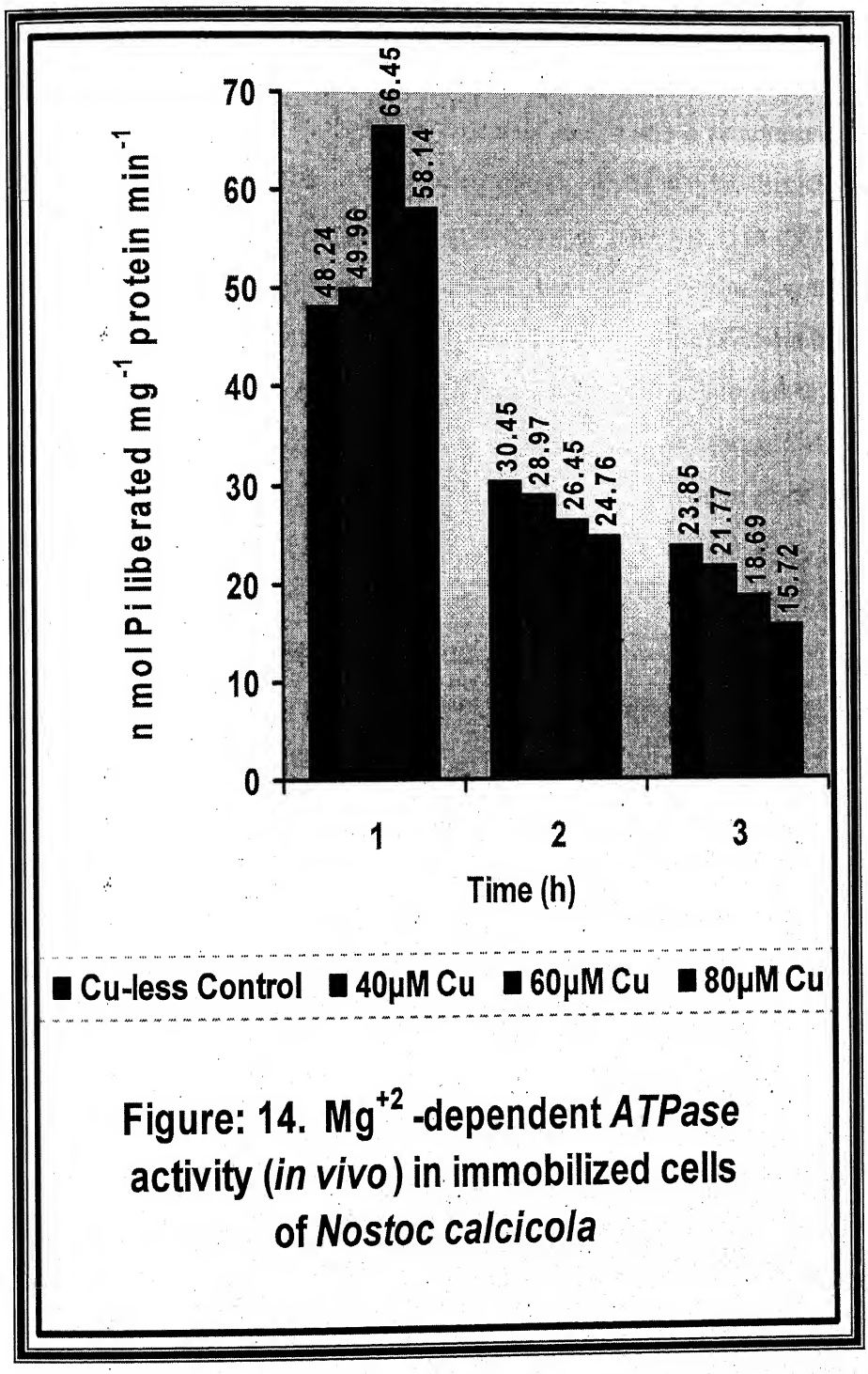

Figure: 14. Mg^{+2} -dependent *ATPase* activity (*in vivo*) in immobilized cells of *Nostoc calcicola*

The effect of Cu on Ca^{2+}-dependent *ATPase* activity (*in vivo*) in free cells is shown in **Figure: 15.** Cu-less control cells showed a higher level (103.21 n mol Pi liberated mg^{-1} protein min^{-1}) at 3 h as compared to Mg^{2+}-dependent *ATPase* activity (*in vivo*). 40μM Cu in this case caused slight enhancement in enzyme activity in 1 h (105.21 n mol Pi liberated mg^{-1} protein min^{-1}) followed by 50% inhibition (54.77 n mol Pi liberated mg^{-1} protein min^{-1}) at 2 h and 60% inhibition (37.52 n mol Pi liberated mg^{-1} protein min^{-1}). The enzyme activity was found to be reduced with respect to concentration and increment in exposure time with a minimum value (28.16 n mol Pi liberated mg^{-1} protein min^{-1}) in 80μM Cu-treated free cells at 3 h. Ca^{2+}-dependent *ATPase* activity (*in vivo*) in immobilized cells **(Figure: 16)** treated with various Cu-concentrations (0-80μM) showed a similar results as presented by its free cells counterpart but it is interesting to note that the inhibition by Cu is always in this case.

The effects of Cu on the enzyme activity under free and immobilized cells were also studied in crude enzyme extract. In this case, the enzyme was harvested from two sets of cells and examined the Cu effect on *in vitro* enzyme activity. Cu-stimulation / inhibition of the *in vitro* Mg^{2+}-dependent *ATPase* activity in free cells **(Figure: 17)** and immobilized cells **(Figure: 18),** were showed almost similar pattern as shown by *in vivo* free cells. However, the level of enzyme activity in present case was slightly higher compared to previous one.

Similar results were obtained in case of *in vitro* Ca^{2+}-dependent *ATPase* activity in free cells **(Figure: 19)** and immobilized cells **(Figure: 20).**

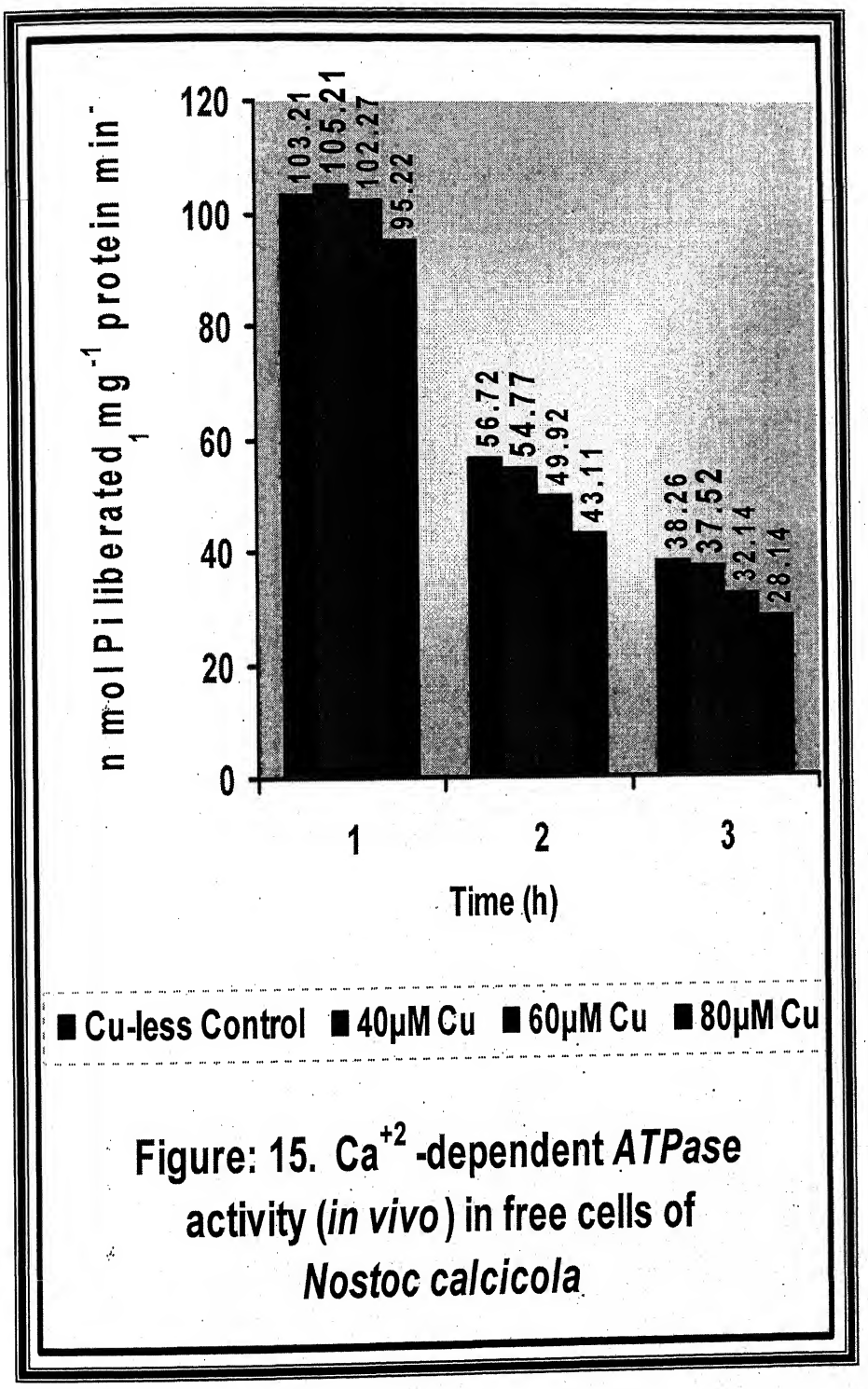

Figure: 15. Ca^{+2} -dependent *ATPase* activity (*in vivo*) in free cells of *Nostoc calcicola*

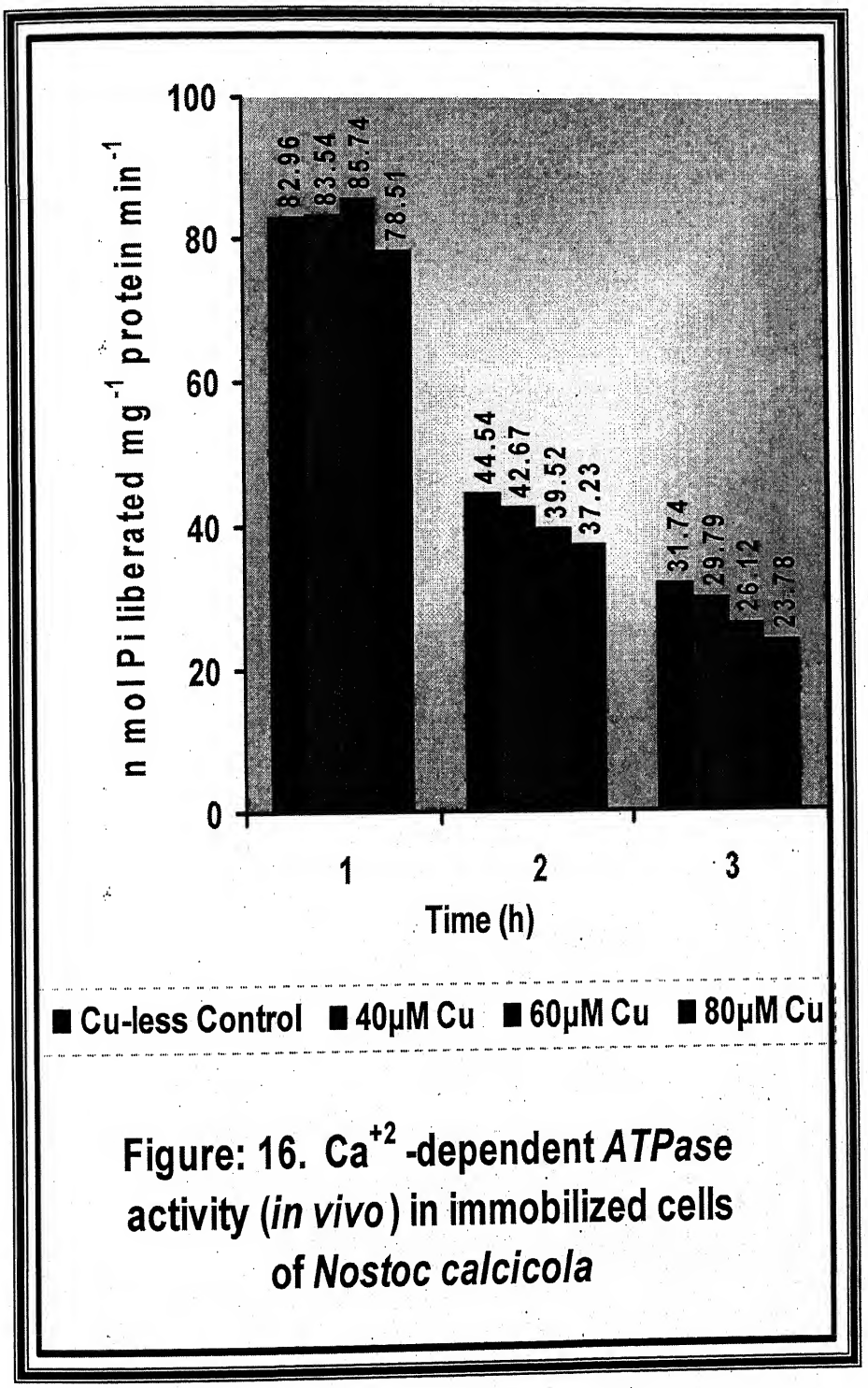

Figure: 16. Ca^{+2} -dependent *ATPase* activity (*in vivo*) in immobilized cells of *Nostoc calcicola*

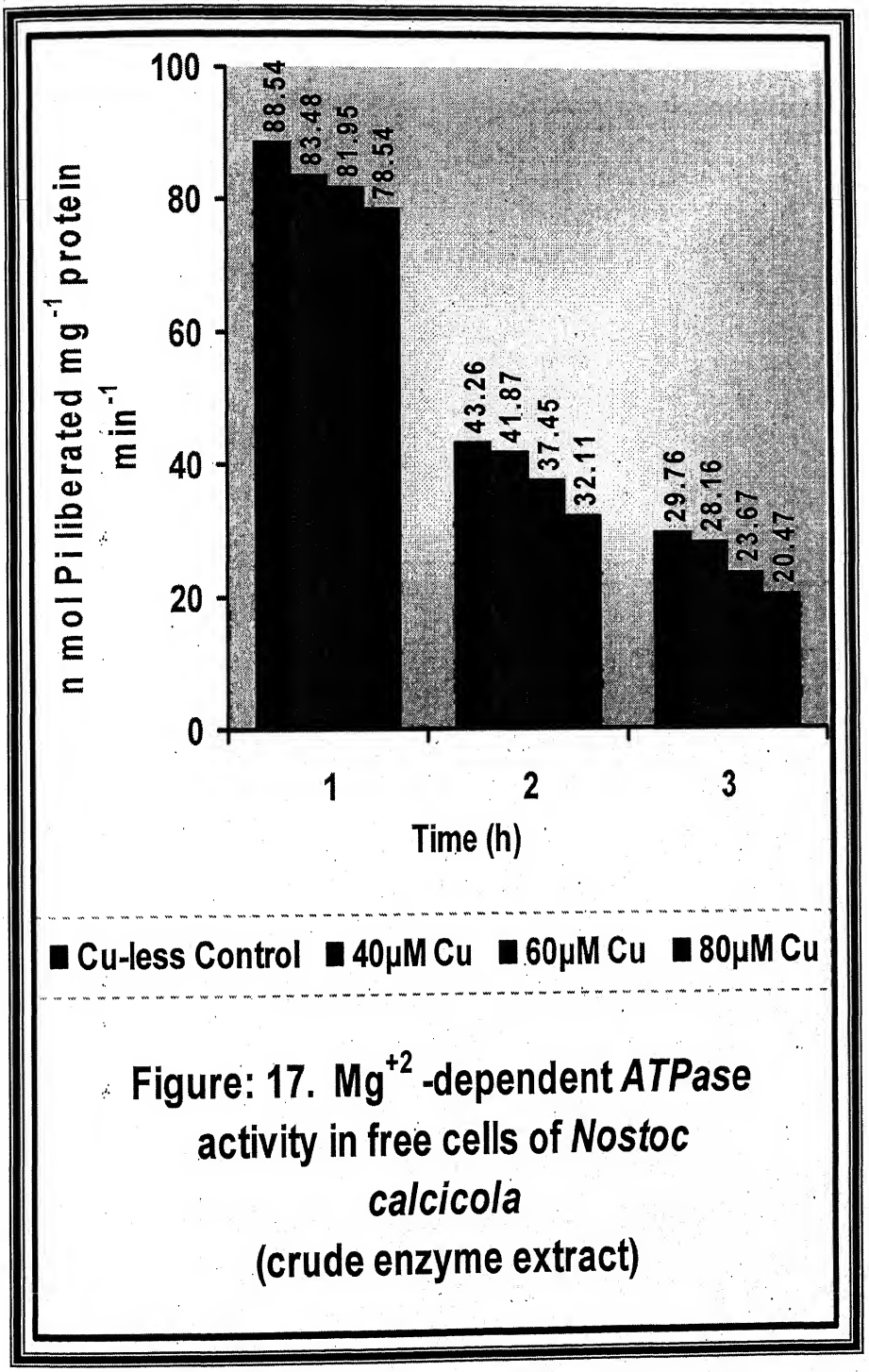

Figure: 17. Mg^{+2} -dependent *ATPase* activity in free cells of *Nostoc calcicola* (crude enzyme extract)

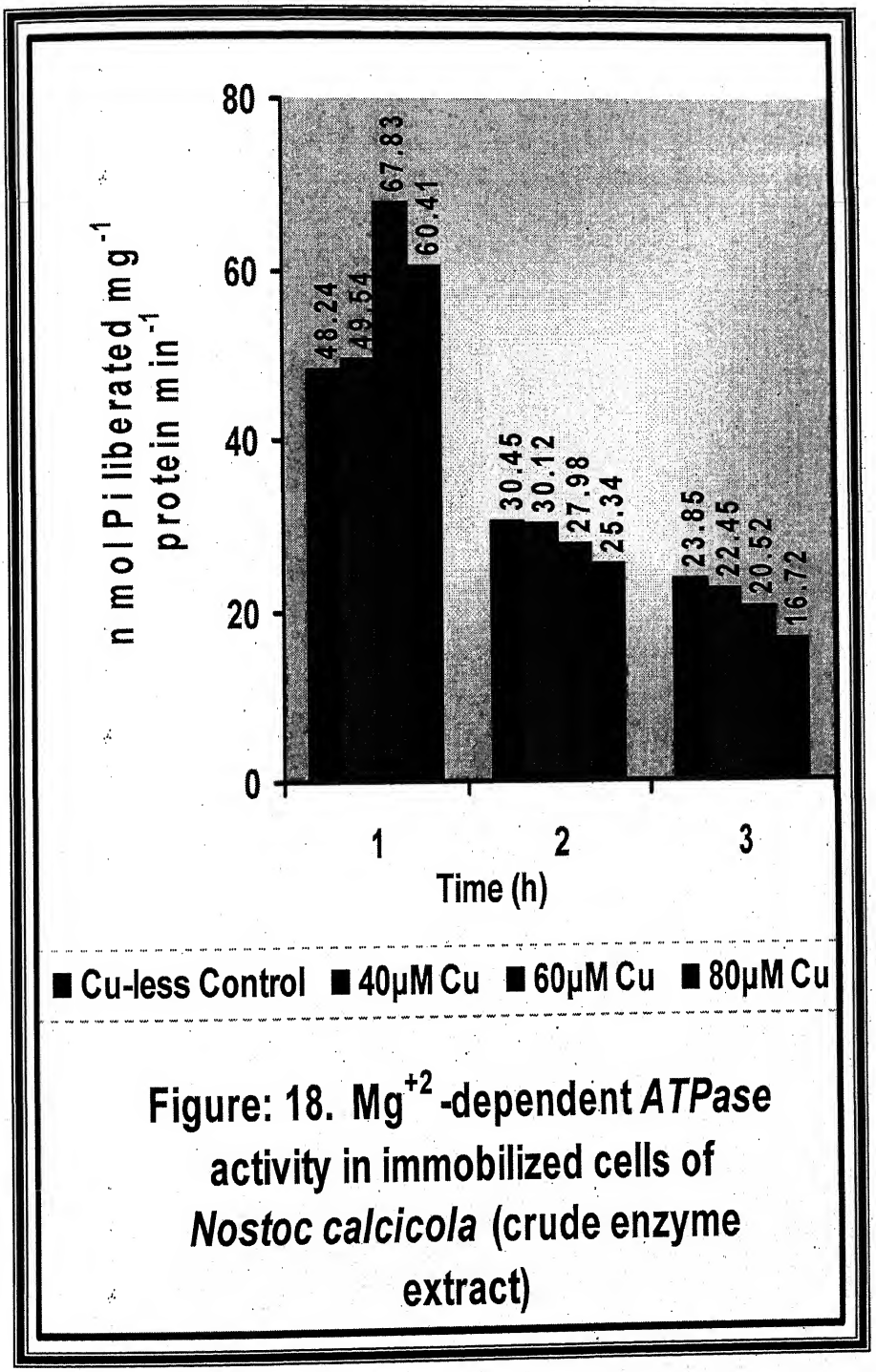

Figure: 18. Mg^{+2}-dependent *ATPase* activity in immobilized cells of *Nostoc calcicola* (crude enzyme extract)

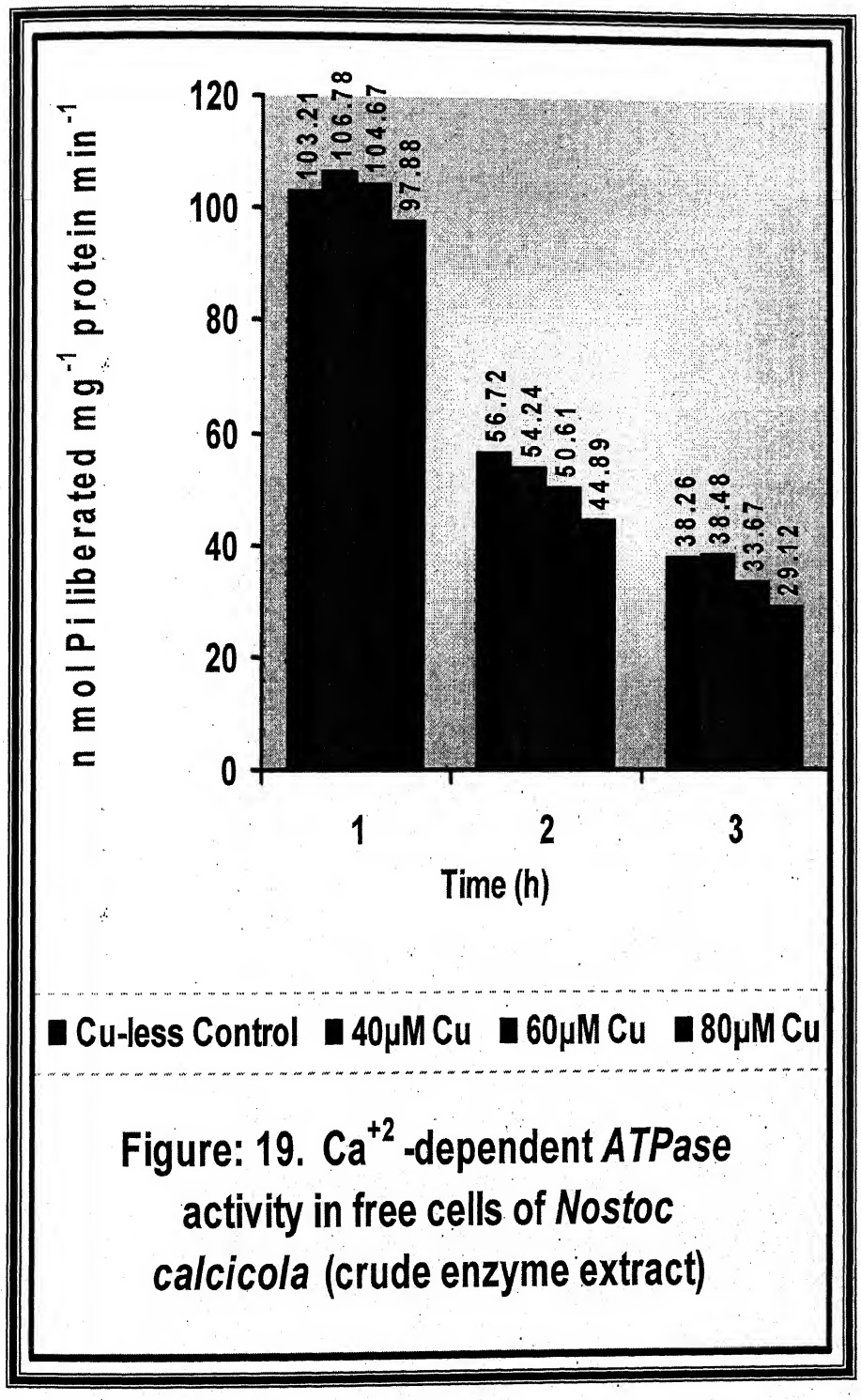

Figure: 19. Ca^{+2} -dependent *ATPase* activity in free cells of *Nostoc calcicola* (crude enzyme extract)

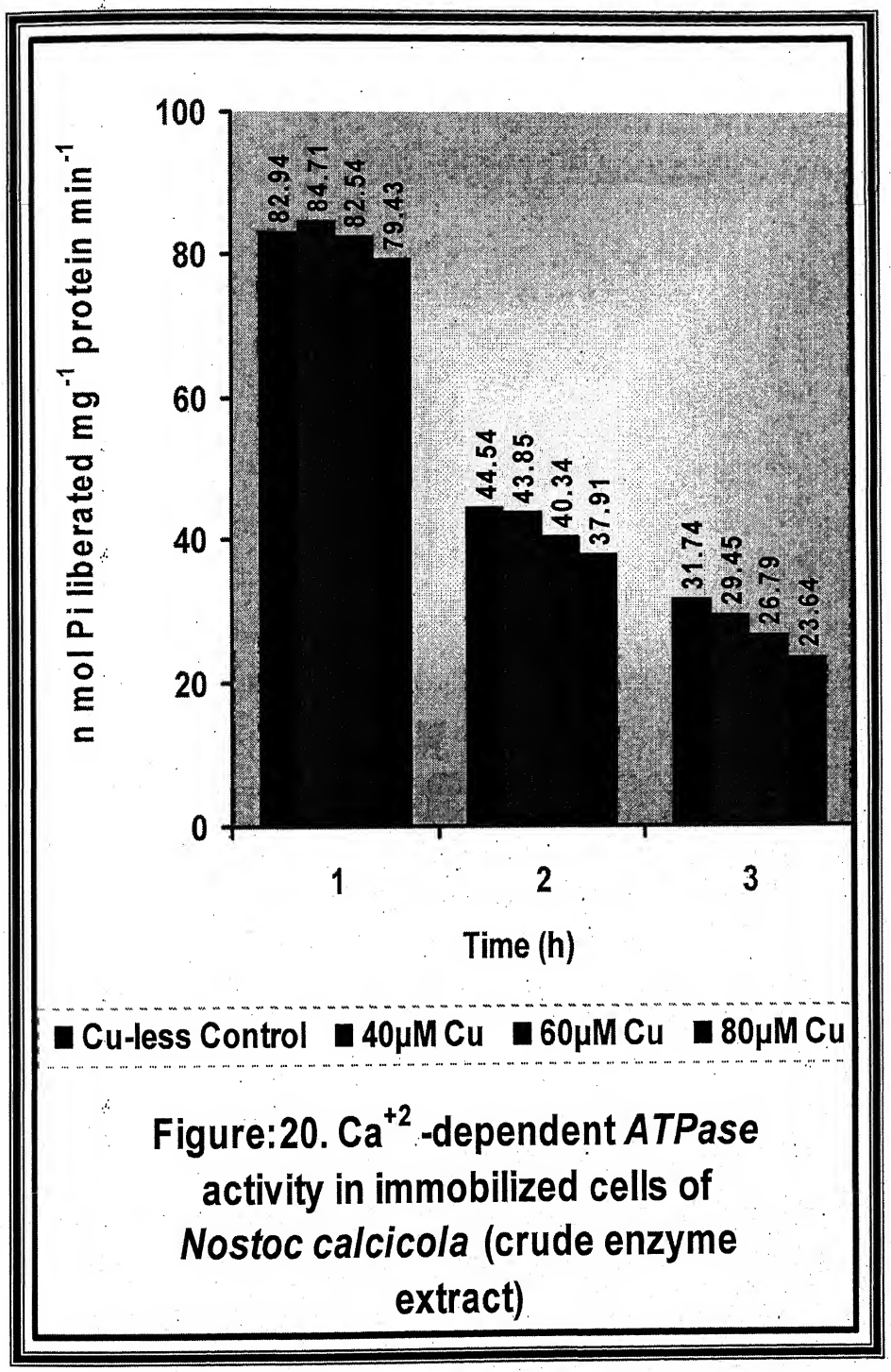

Figure:20. Ca^{+2}-dependent *ATPase* activity in immobilized cells of *Nostoc calcicola* (crude enzyme extract)

5.4. INFLUENCE OF COPPER ON THE *IN VIVO* ACTIVITY OF *NITROGENASE* AND *GLUTAMINE SYNTHETASE* (*TRANSFERASE*):-

5.4.1. INFLUENCE OF COPPER ON THE *IN VIVO* ACTIVITY OF *NITROGENASE*:-

The physiological superiority of immobilized cells over free cells was also put to test with reference to *nitrogenase* activity (C_2H_2 / C_2H_4 assay). The metal-less free cells showed increasing trends in the enzyme activity attaining a maximum of 6.12 n mol C_2H_4 produced mg^{-1} protein min^{-1} **(Figure: 21).** The Cu concentration (60µM) brought about 26% decline in total value of *nitrogenase* activity (at 3 h) and a lowering trend could be seen even-after 30 min exposure of cells to 60µM Cu. Free cells reflected enhanced levels of inhibition by Cu in a more or less concentration-dependent manner (100 to 500µM Cu). Cells dosed with 500µM Cu showed maximum inhibition (91%) in enzyme activity.

The immobilized cells, like in other cases, also showed 1.3 times higher levels of *nitrogense* activity over free cells and the resistance against *nitrogenase* inhibition by Cu **(Figure: 22).** The Cu concentration (60µM) could bring about lesser extent of inhibition of *nitrogenase* activity as compared to free cells (10%). However the high concentration of Cu (200 to 500µM) could bring similar extent of reduction with 12% of the enzyme activity still available during 3 h exposure of cells in such a state.

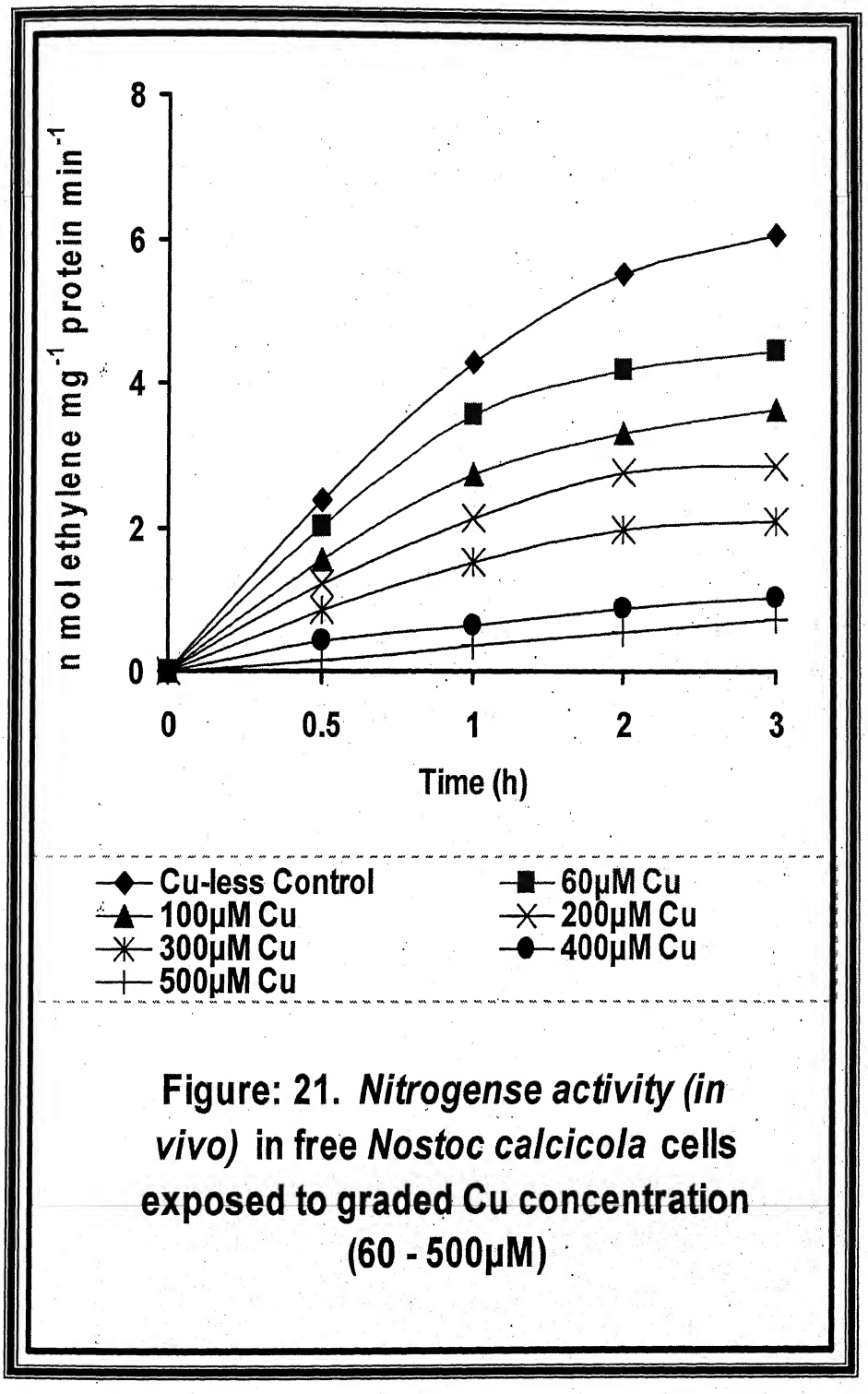

Figure: 21. ***Nitrogense activity (in vivo)*** **in free** ***Nostoc calcicola*** **cells exposed to graded Cu concentration (60 - 500µM)**

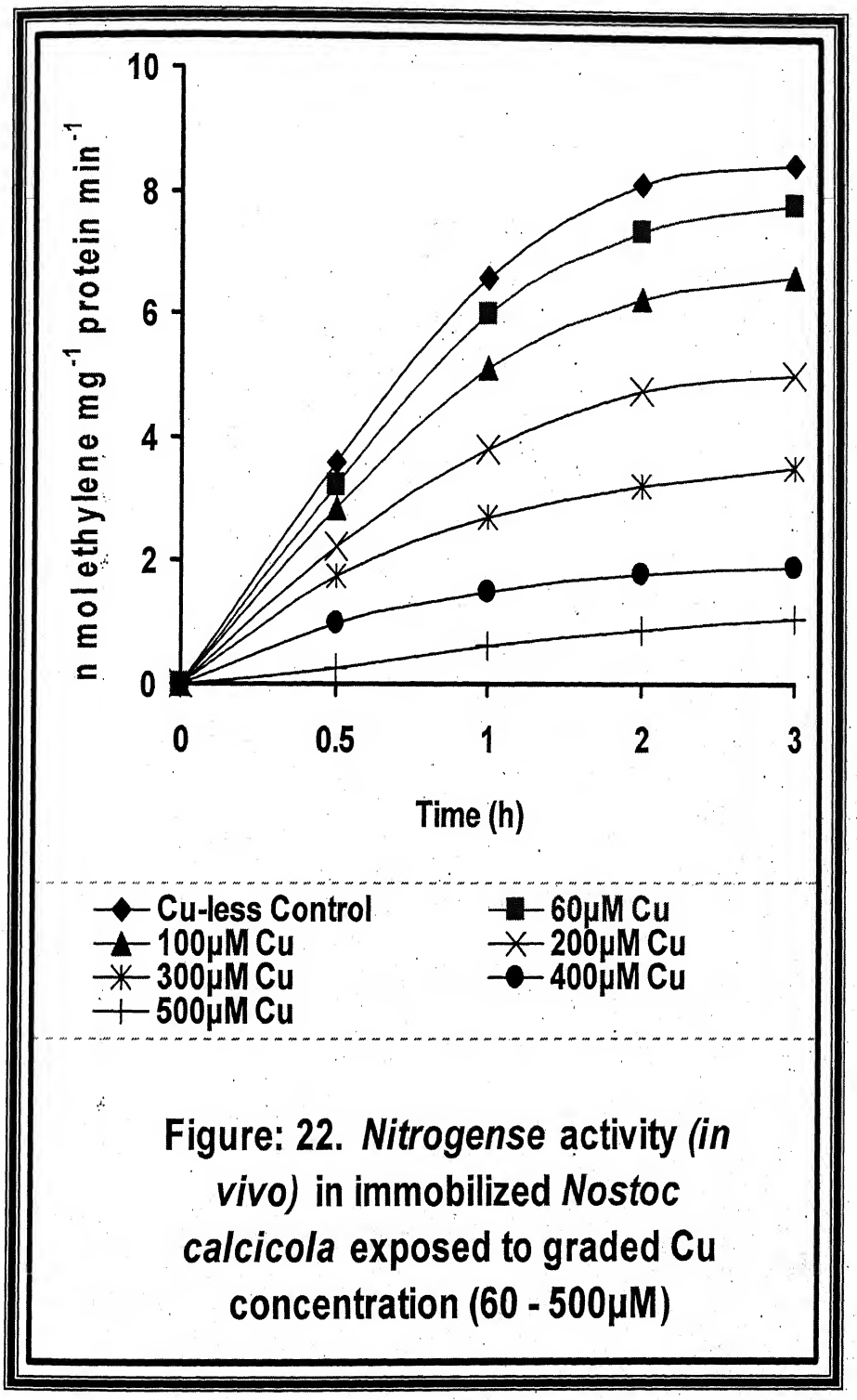

Figure: 22. *Nitrogense* activity *(in vivo)* in immobilized *Nostoc calcicola* exposed to graded Cu concentration (60 - 500µM)

5.4.2. INFLUENCE OF COPPER ON THE *IN VIVO* ACTIVITY OF *GLUTAMINE SYNTHETASE* (*TRANSFERASE*):-

As the previous experiments revealed *nitrogenase* sensitivity towards Cu, it was of interest to look for the metal sensitivity of *glutamine synthetase,* the key enzyme in ammonium assimilation in such prokaryotes. It may be mentioned that only *glutamine synthetase* (*transferase*) activity and not the biosynthetic, has been studied in the present case. Free cells had the basal level of enzyme activity amounting to 1.45μ mol γ-glutamylhydroxamate mg^{-1} protein min^{-1} with a maximum of 2.4μ mol γ-glutamylhydroxamate mg^{-1} protein min^{-1} (at 3 h). It was interesting to note the Cu resistance of the enzyme during 1 h exposure even if the metal concentration increased by a factor of 5 from the initial 60μM Cu concentration. It was only after this duration that inhibitions by 100μM Cu or 200μM Cu became quite distinct. The subsequent increase in Cu concentration to 300μM, inhibited enzyme activity by 22% even at 30 min exposure, and the extent of inhibition approximated 35% with 500μM Cu within the same duration **(Figure: 23).** The decline in *glutamine synthetase* (*transferase*) activity followed almost an exponential route for both these metal concentrations, but never touched zero even-after 3 h.

Immobilized cells, as expected, were characterized by a 70% increase in the basal level activity of *glutamine synthetase* compared to free cells. Also, the ultimate gain in terms of enzyme activity during experimental regime touched 3.64μ mol γ-glutamylhydroxamate mg^{-1} protein min^{-1}.

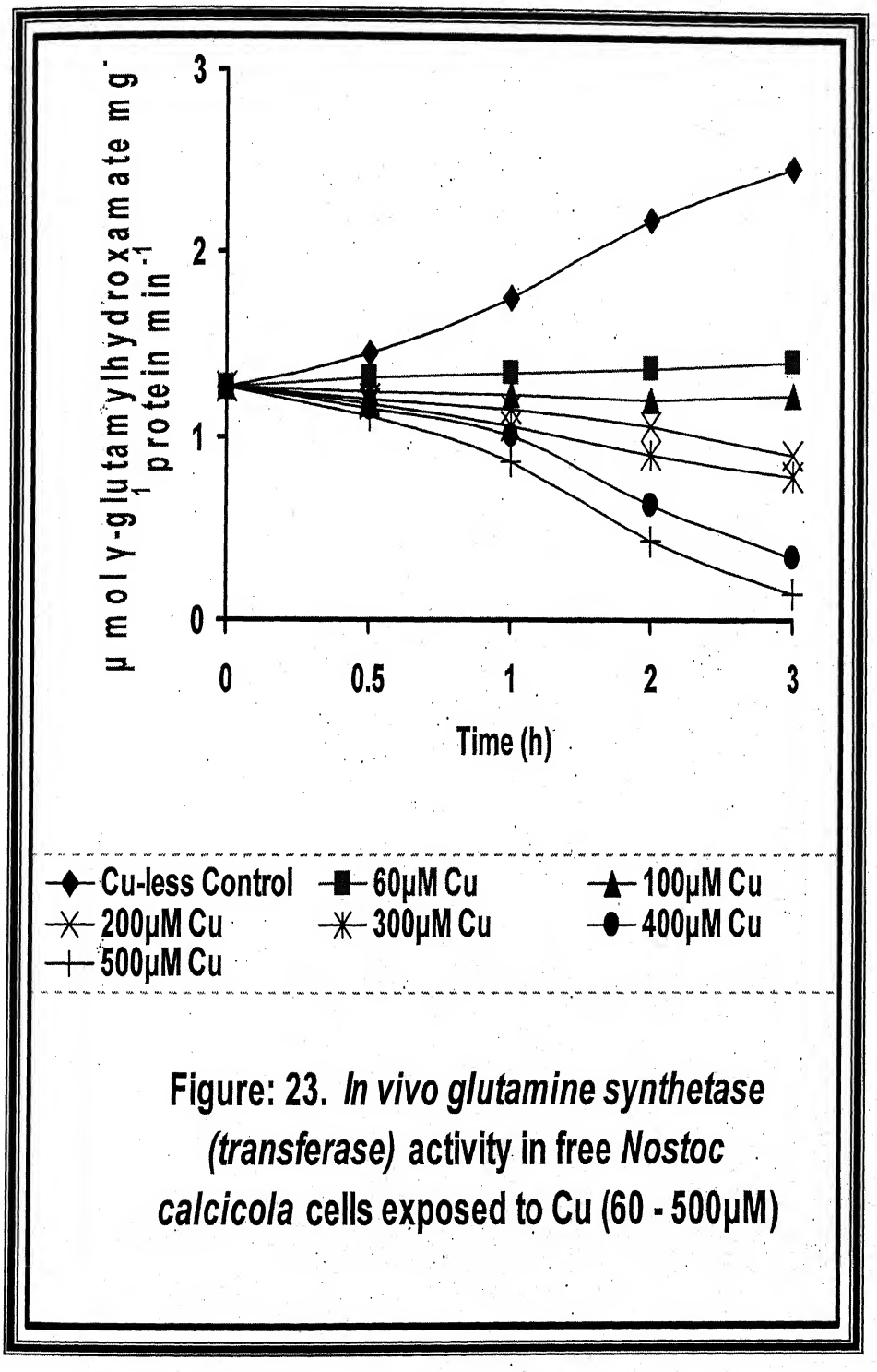

Figure: 23. ***In vivo glutamine synthetase (transferase)*** **activity in free** ***Nostoc calcicola*** **cells exposed to Cu (60 - 500µM)**

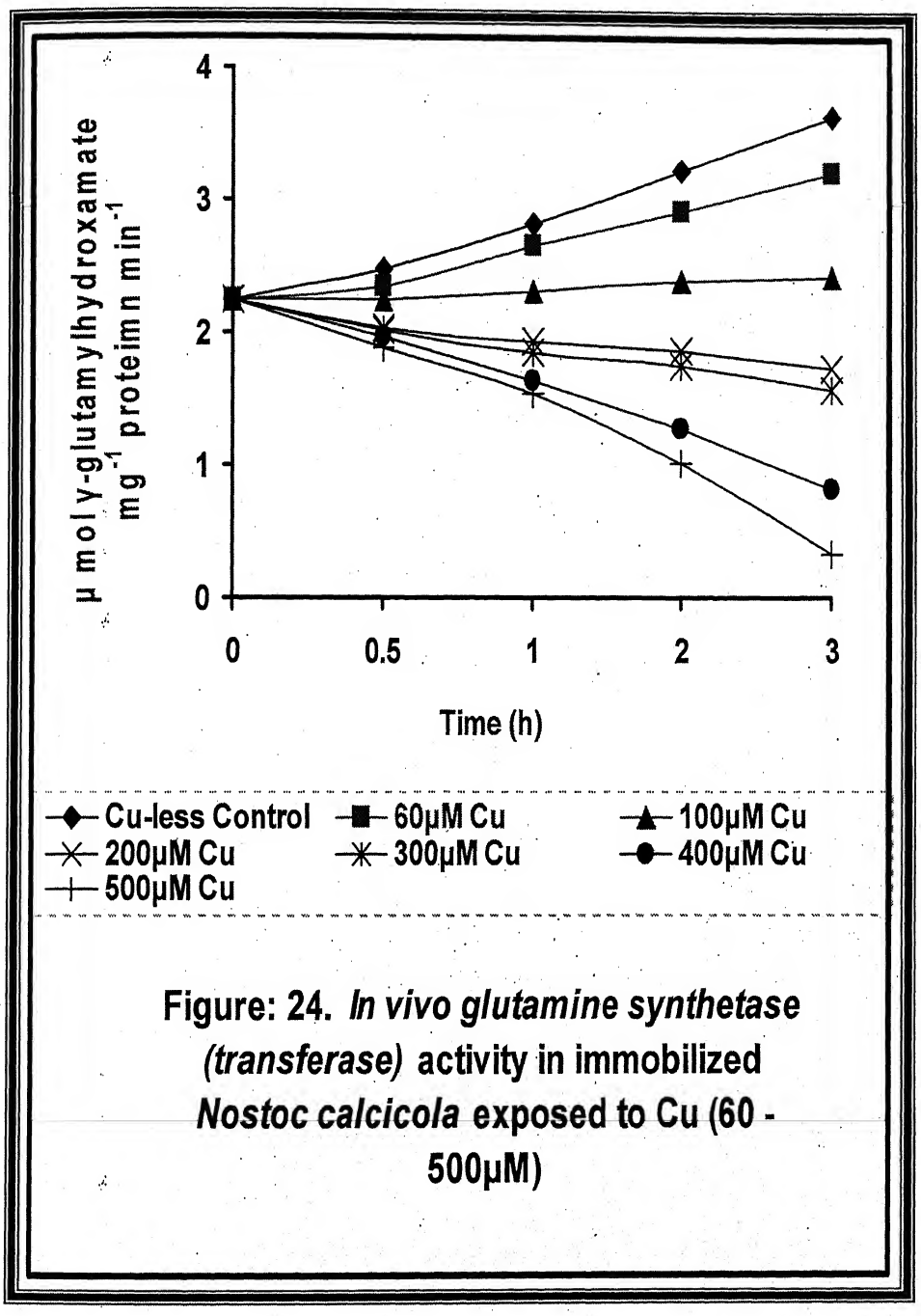

Figure: 24. ***In vivo glutamine synthetase (transferase)*** **activity in immobilized** ***Nostoc calcicola*** **exposed to Cu (60 - 500µM)**

The close proximity between points (at 2 h) for metal-less control and beads exposed to 60μM, suggests that enzyme under such state of cells can resist Cu inhibition quite efficiently with a major decline in case the duration of exposure increased any further. Cu (60μM) that was the saturating concentration for immobilized cells, could bring about a maximum of 13% inhibition of enzyme activity with respect to Cu-less control beads even-after 3 h of metal exposure. Subsequent increase in the Cu concentration (300 to 500μM) inhibited the enzyme activity depending on the Cu concentration as well as the duration of exposure. For immobilized cells also, a zero activity of the enzyme could not be achieved within the tested Cu concentration range (60 to 500μM) and the time of exposure as well **(Figure: 24).**

5.5. $^{14}CO_2$ - INCORPORATION:-

A comparison of Cu uptake pattern between free and immobilized cells established that cells in this condition showed saturation at 3 h with a maximum $^{14}CO_2$-incopration (13580 CPM mg^{-1} protein in Cu-less control cells **(Figure: 25).** The increased efficiency of Cu uptake by immobilized cells formed the basis of comparison of $^{14}CO_2$-uptake with the aim of knowing about the vital aspect of photoautotrophy in cyanobacterial cells in free and immobilized state. **Figure: 25** shows the pattern of $^{14}CO_2$-incorporation in free cells dosed with a much lower Cu concentration (30μM) as well as 60μM Cu concentration for metal uptake. The control cells showed increasing trend of $^{14}CO_2$-uptake with a maximum of 13580 CPM mg^{-1} protein (at 3 h) followed by an almost 50% reduction at 30μM Cu.

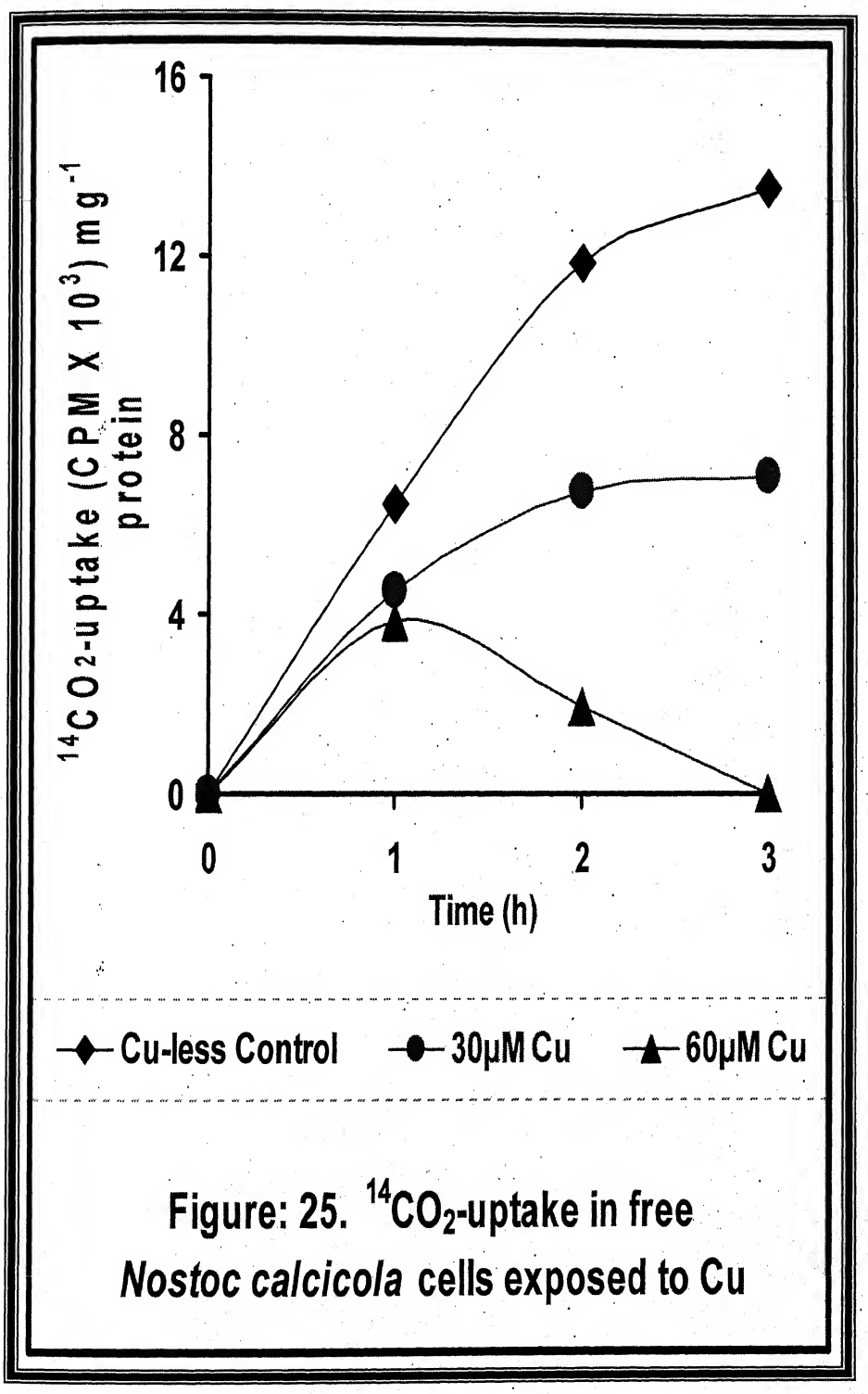

Figure: 25. $^{14}CO_2$-uptake in free *Nostoc calcicola* cells exposed to Cu

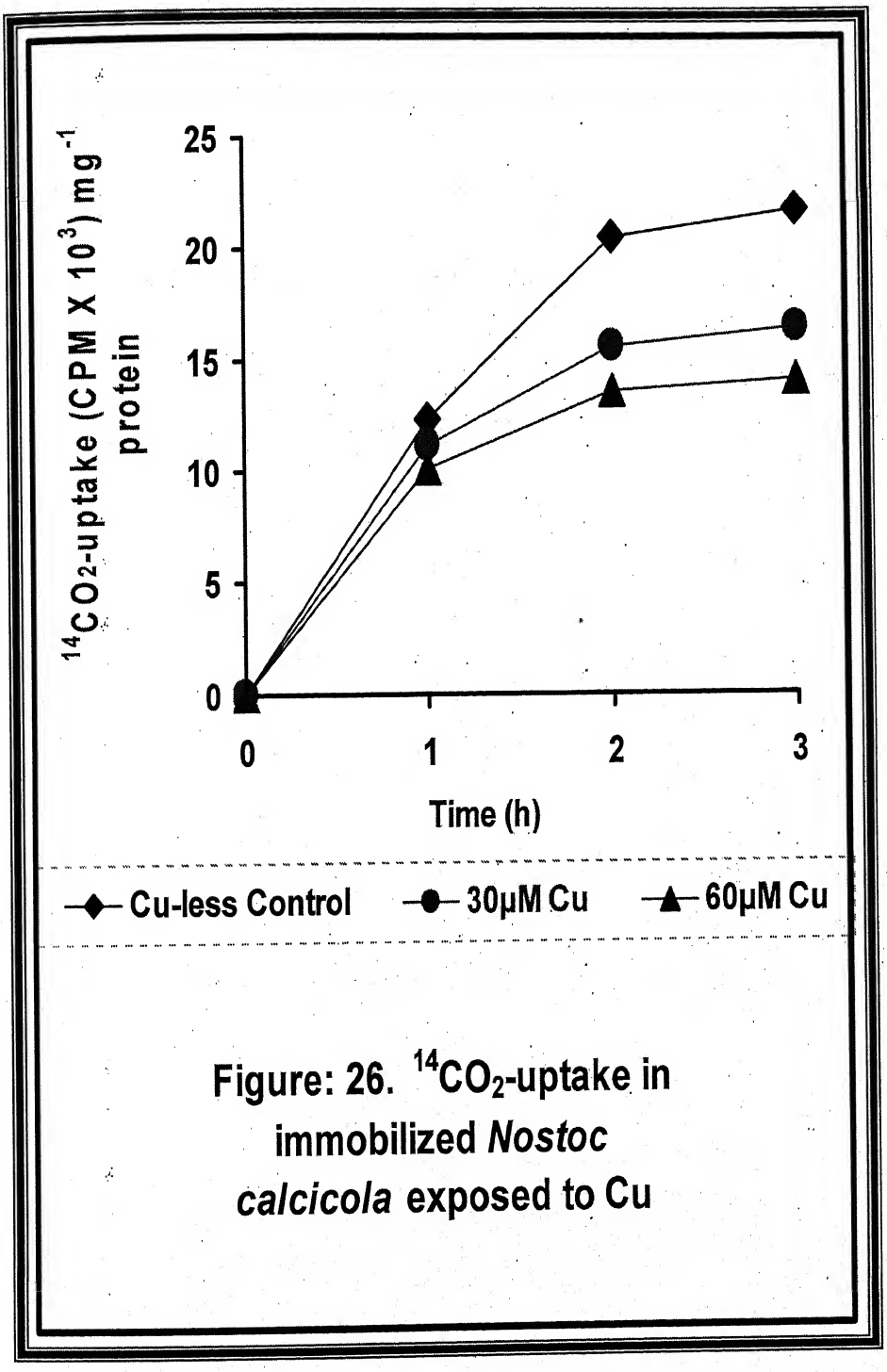

Figure: 26. $^{14}CO_2$-uptake in immobilized *Nostoc calcicola* exposed to Cu

Such free cells, if dosed with the 80μM Cu concentration, showed decreased level of $^{14}CO_2$-uptake only during 1-3 h incubation with complete abolition in $^{14}CO_2$-uptake at 3 h.

The immobilized cells growing as control were about 25% more efficient in $^{14}CO_2$-uptake compared to their free cell counterpart (compared at 3 h; **Figure: 26).** The immobilized cells were more efficient in $^{14}CO_2$-incorporation at 30μM Cu as compared to their respective free cells. It was interesting to note the increased extent of $^{14}CO_2$-incorporation in immobilized cells dosed with 60μM Cu as there was only 33% inhibition compared to 55% inhibition of the process in free cells exposed to the corresponding Cu concentration (80μM). Such comparisons are based on the final values of $^{14}CO_2$-uptake at 3 h.

5.6. STUDIES ON COPPER TOLERANT / RESISTANT STRAIN OF *Nostoc calcicola*:-

5.6.1. GROWTH BEHAVIOR OF COPPER SENSITIVE (Cu^s) AND COPPER TOLERANT (Cu^r) STRAIN :-

The general growth of Cu-sensitive (Cu^s) and Cu-tolerant (Cu^r) strain has been summarized in **Figure: 27.** The Cu-less control cells of Cu^s showed sigmoid curve ~~with a *K* of 0.046~~. As the lowest curve indicates 5μM Cu to be lethal for Cu^s strain, Cu^r strain was grown in 70μM Cu, and also as Cu-less control. Characteristically, the Cu^r grows at a lower pace compared to Cu^s even in the Cu-less medium ~~(*K*= 0.038)~~. The overall growth behavior of Cu^r in 70μM Cu showed considerable metal tolerance ~~(*K*= 0.036)~~.

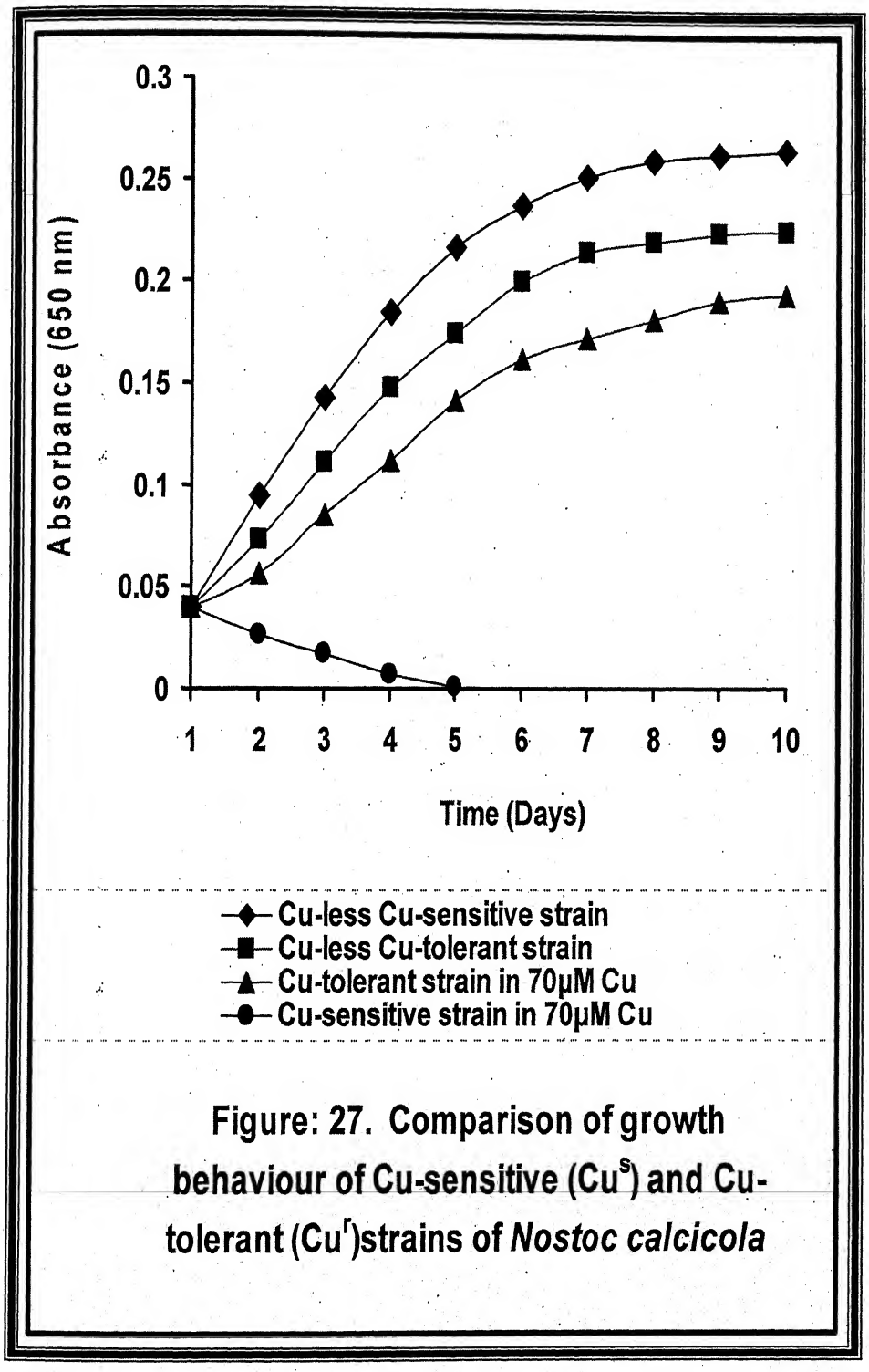

Figure: 27. Comparison of growth behaviour of Cu-sensitive (Cu^S) and Cu-tolerant (Cu^r)strains of *Nostoc calcicola*

5.6.2. COPPER UPTAKE IN Cu^r STRAIN:-

After the growth behaviour of Cu^r strain in 70μM Cu, It was interested to look for the possible alteration(s) in Cu-uptake patterns in Cu^s and Cu^r strains of *Nostoc calcicola*. **Figure: 28** summarizes Cu uptake pattern for Cu^s and Cu^r strains taking 60μM Cu as the common metal dose. As both the strains showed time-dependent metal uptake. Cu^r strain accumulated 72.15 n mol Cu mg^{-1} protein, during 1 h compared to higher values for the Cu^s strain (96.89n mol Cu mg^{-1} protein) or in other words, accumulating to 26% less intake in the former. Such a narrow difference prompted me to look into the other possible mechanism related to Cu-tolerance in Cu^r and the monitoring of Cu-efflux was the foremost.

5.6.3. COPPER EFFLUX IN Cu^s AND Cu^r STRAINS:-

Figure: 29 accommodates data on Cu-efflux from the Cu-fed Cu^s and Cu^r strains of *Nostoc calcicola*. The cyanobacterial cells in both the cases, dosed with 60μM Cu for 1 h and re-suspended in phosphate buffer (0.01M; pH 8.0) after repeated EDTA (10μM) washing (for removal of adsorbed Cu). The concentration of Cu in buffer accounted for the metal amount extruded from either Cu^s or Cu^r cells whereas Cu^s cells did not show any Cu-efflux. The Cu^r cells followed a time dependent Cu-efflux. The linearity of curve shows that metal efflux ratio was higher (2.42 n mol Cu mg^{-1} protein min^{-1}) for the first 20 min, followed by slow efflux up to 57.12 n mol Cu mg^{-1} cell protein. However, the extent of Cu extrusion, in Cu^r strain was in close proximity with the amount taken up by such cells during uptake studies lasting 1 h.

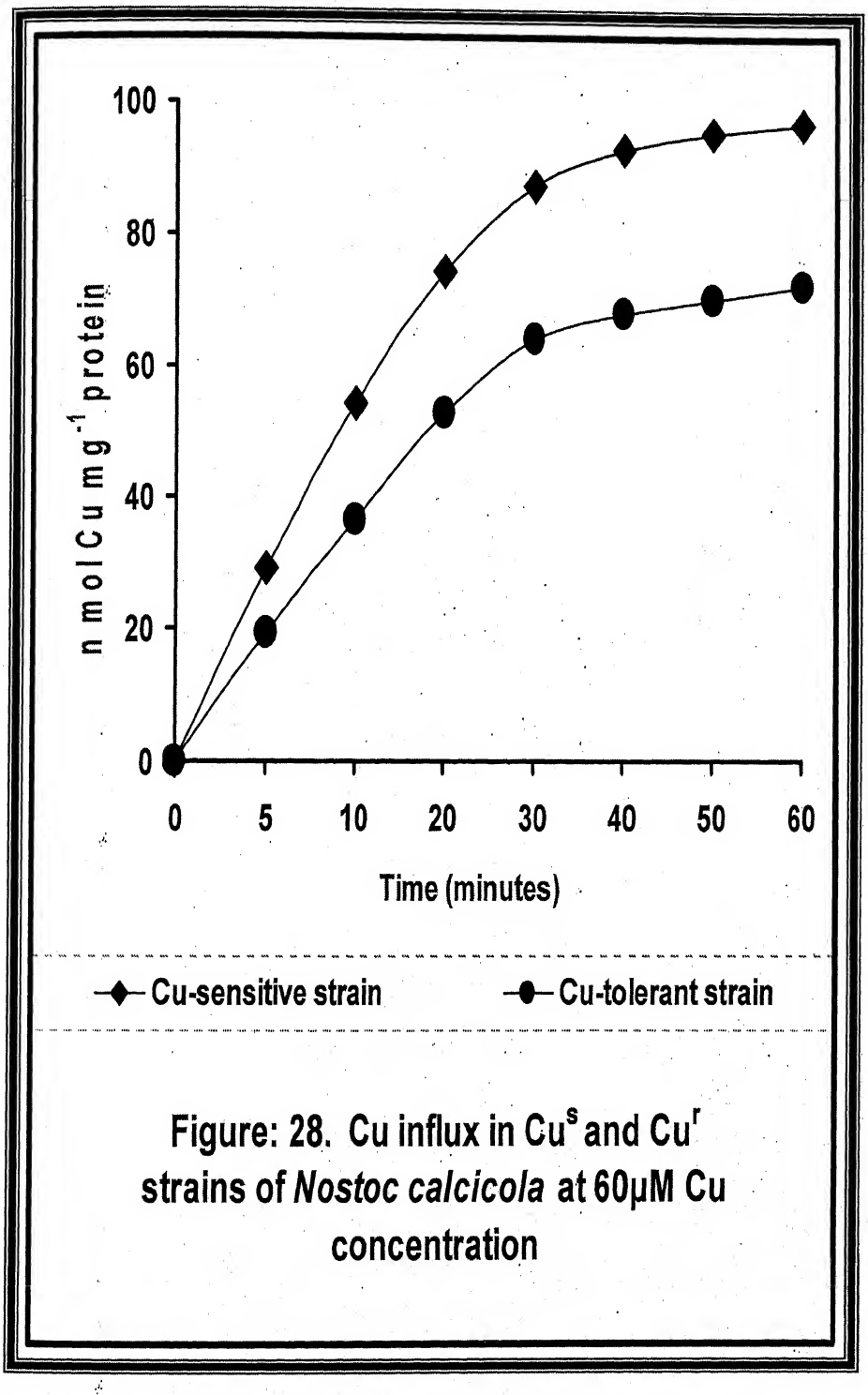

Figure: 28. Cu influx in Cu^s and Cu^r strains of *Nostoc calcicola* at 60µM Cu concentration

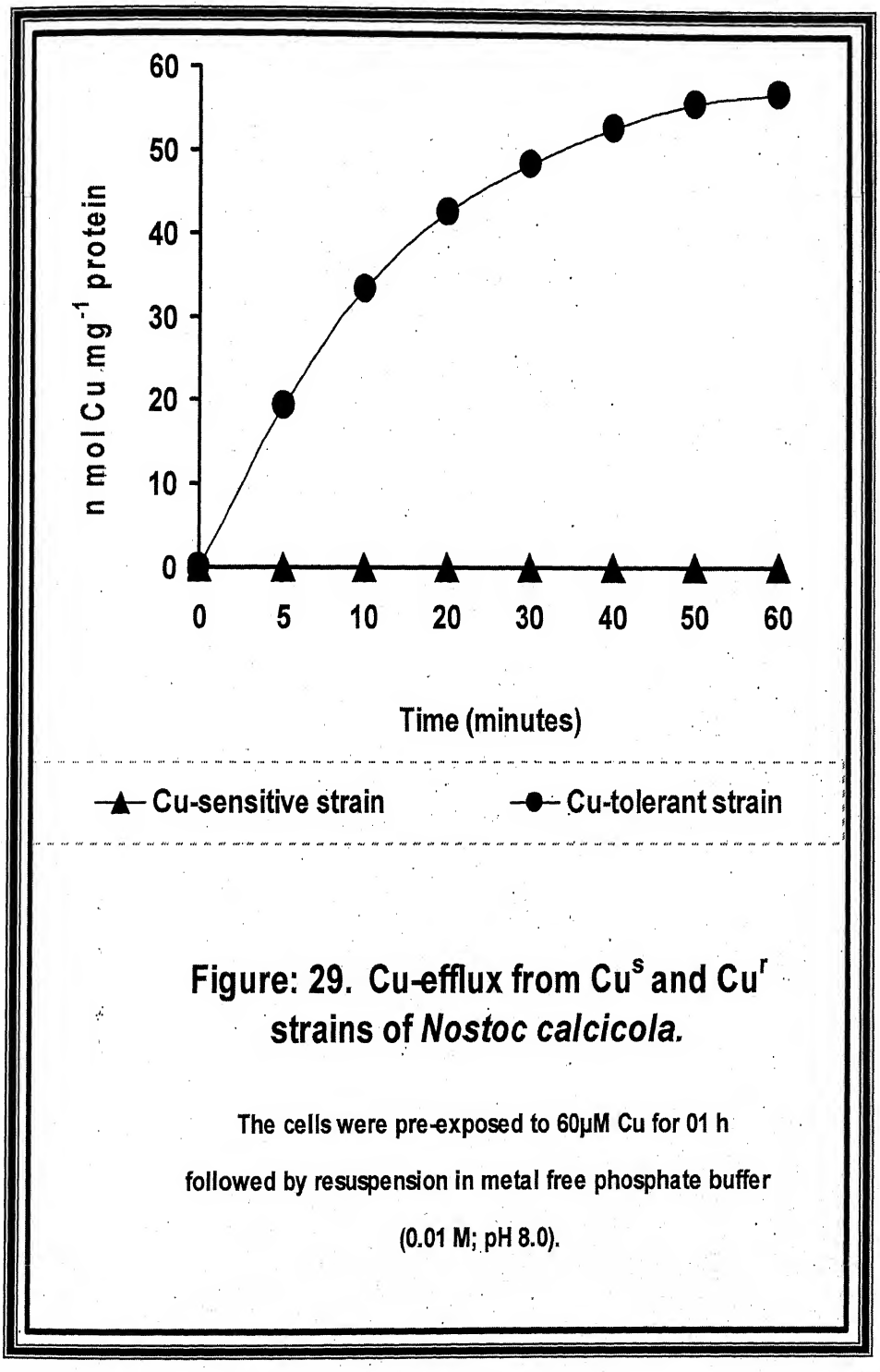

Figure: 29. Cu-efflux from Cu^s and Cu^r strains of *Nostoc calcicola.*

The cells were pre-exposed to 60µM Cu for 01 h followed by resuspension in metal free phosphate buffer (0.01 M; pH 8.0).

5.7. FACTORS REGULATING COPPER EFFLUX IN Cu^r STRAIN OF *Nostoc calcicola* :-

5.7.1. LIGHT AND EXOGENOUS ATP REGULATION OF COPPER EFFLUX IN Cu^r STRAIN:-

It is well established from previous experiments that metal uptake in photoautotroph, is regulated to major extent, by light-generated energy. Therefore, the present experiments account for the possible regulation(s) of Cu-efflux by light or the exogenous ATP. As the dark incubated Cu^r cells did not show significant Cu-efflux (only 3.15 n mol Cu mg^{-1} protein at 1 h; **Figure: 30**), the crucial role of light generated energy in the efflux mechanism, was indicated. In case, such cells were added with 10μM ATP, there was significant Cu-efflux (30.11 n mol Cu mg^{-1} protein; 1 h). This value is more than 10-fold over the dark incubated Cu^r cells devoid of exogenous ATP. Cu^r strain did not show significant increase in Cu-efflux in terms of rate or the extent when grown in light + ATP over the light grown cells lacking ATP. Such a trend, therefore, indicates that light generated energy was sufficient enough to drive Cu-efflux in Cu^r strain.

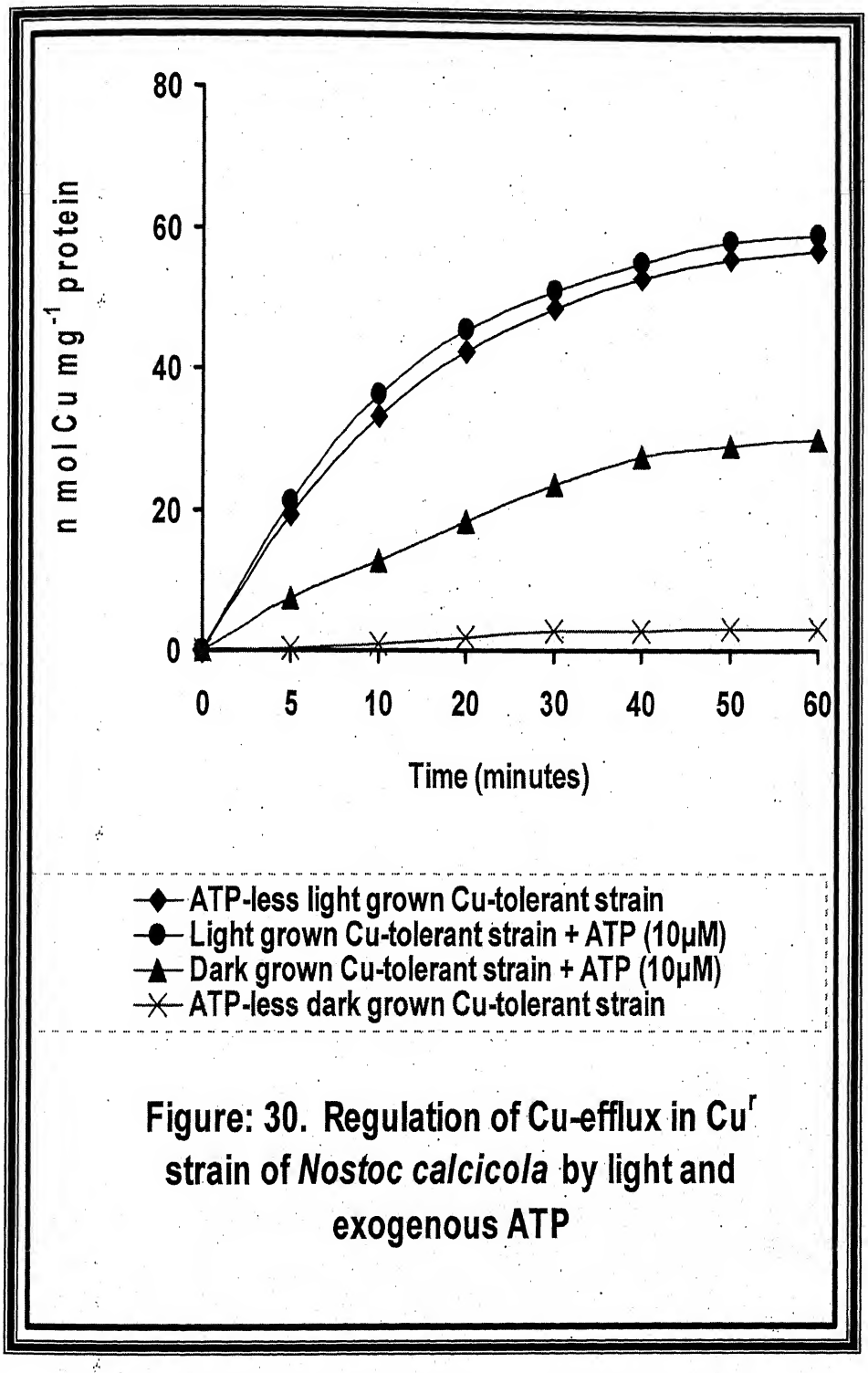

Figure: 30. Regulation of Cu-efflux in Cu^r strain of *Nostoc calcicola* by light and exogenous ATP

5.7.2. REGULATION OF COPPER EFFLUX IN Cu^r STRAIN OF *Nostoc calcicola* BY DIFFERENT METABOLIC INHIBITORS / UNCOUPLERS:-

The relative contribution of different metabolic inhibitors / uncouplers in regulating Cu-efflux in Cu^r strain of *Nostoc calcicola* is shown in **Figure: 31.**

The Cu-efflux inhibited by mercaptoethanol which reduced Cu-efflux by 25% (39.52 n mol Cu mg^{-1} protein).

Sodium azide (10μM) inhibits respiratory electron transport and uncouples oxidative phosphorylation; caused approximately 50% inhibition of Cu-efflux.

Cu-efflux was also influenced by DCCD which inhibited 75% Cu-efflux. On the other hand Cu-efflux in Cu^r strain was completely inhibited by pCMB (1.0μM).

5.7.3. DILLUTION MEDIATED COPPER EFFLUX IN Cu^r STRAIN:-

Since metal extrusion from the prokaryotic cells is taken as one of the bases of tolerance, experiments were designed to study the extent of Cu extrusion in growth medium added with different Cu dilutions (6, 0.6, 0.24, 0.12, 0.08 and 0.06μM) thus corresponding to ranges of 10, 100, 250, 500, 750 and 1000-fold.

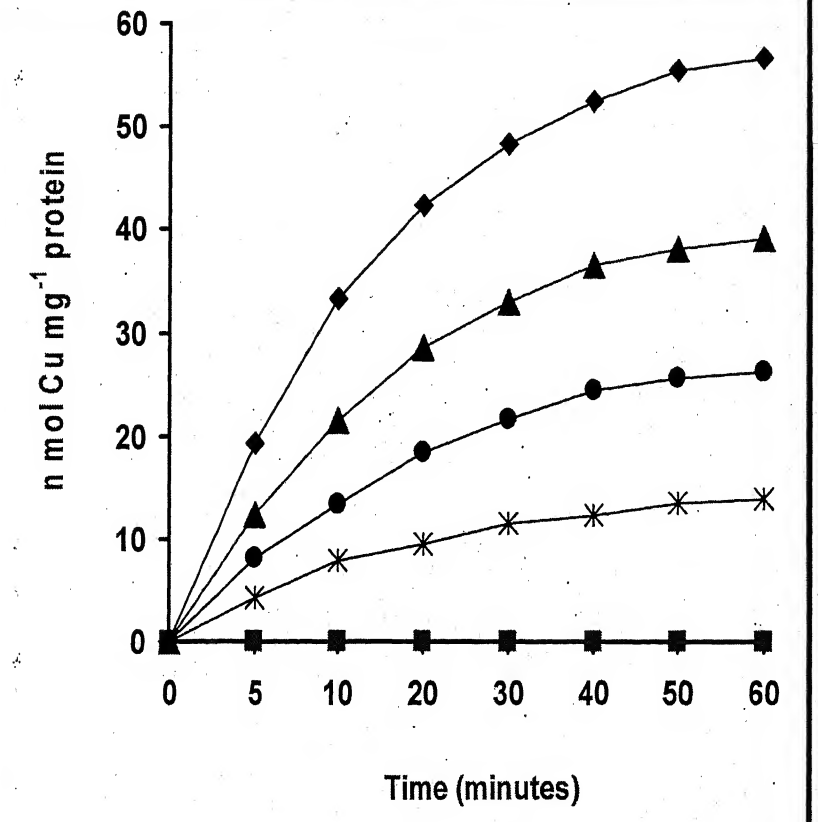

Figure: 31. Regulation of Cu-efflux in Cu^r strain of *Nostoc calcicola* by different Metabolic inhibitors / uncouplers

The results in **Figure: 32** demonstrate the extent of Cu-efflux in Cu-free growth medium and those containing different Cu dilutions. The pattern of Cu extrusion by light grown Cu^r strain showed that the rate as well as the extent of total Cu extrusion was dilution (10-1000-folds) and time (0-60 min) dependent. The uppermost curve indicates that almost entire amount of Cu (72.15 n mol Cu mg^{-1} protein) taken in by the Cu^r strain could be extruded in the Cu-free medium (57.12 n mol Cu mg^{-1} protein). A 10-fold diluted medium, however, showed no Cu extrusion. Also, metal extrusion was only 10.04 n mol Cu mg^{-1} protein for Cu^r strain in 100-fold dilution; a value, almost 6 times lower compared to the Cu-free medium but more with respect to medium with 10-fold dilution as noted earlier. The rate as well as total Cu extrusion increased further dilution (250-fold) reaching 17.79 n mol Cu mg^{-1} protein with a rate of 0.593 n mol Cu mg^{-1} protein min^{-1} (as calculated for the first 20 min). A 500-fold diluted medium gained 30.43 n mol Cu mg^{-1} protein from Cu^r cells accompanied by the increased efflux rate as 1.13 n mol Cu mg^{-1} protein min^{-1} (at 20 min), thus amounting to a 2-fold Cu recovery over the 250-fold dilution sets. Cu extrusion, however, was highest for medium with 1000-fold dilution (0.06μM Cu) with a gain of 54.1 n mol Cu mg^{-1} protein min^{-1}. This amount corresponds to almost 94% of Cu-efflux in a Cu free medium. Similar trials with 750-fold dilution (0.08μM Cu in the medium) also enhanced Cu-efflux as well as total Cu output corresponding to 76% of that in 1000-fold diluted medium. ANOVA clearly established that Cu-efflux was regulated by dilution ($\mathbf{F_{dilution\ 6,30} = 58.86, p < 0.005}$) compared to time ($\mathbf{F_{time\ 5,30} = 8.57, p < 0.05}$).

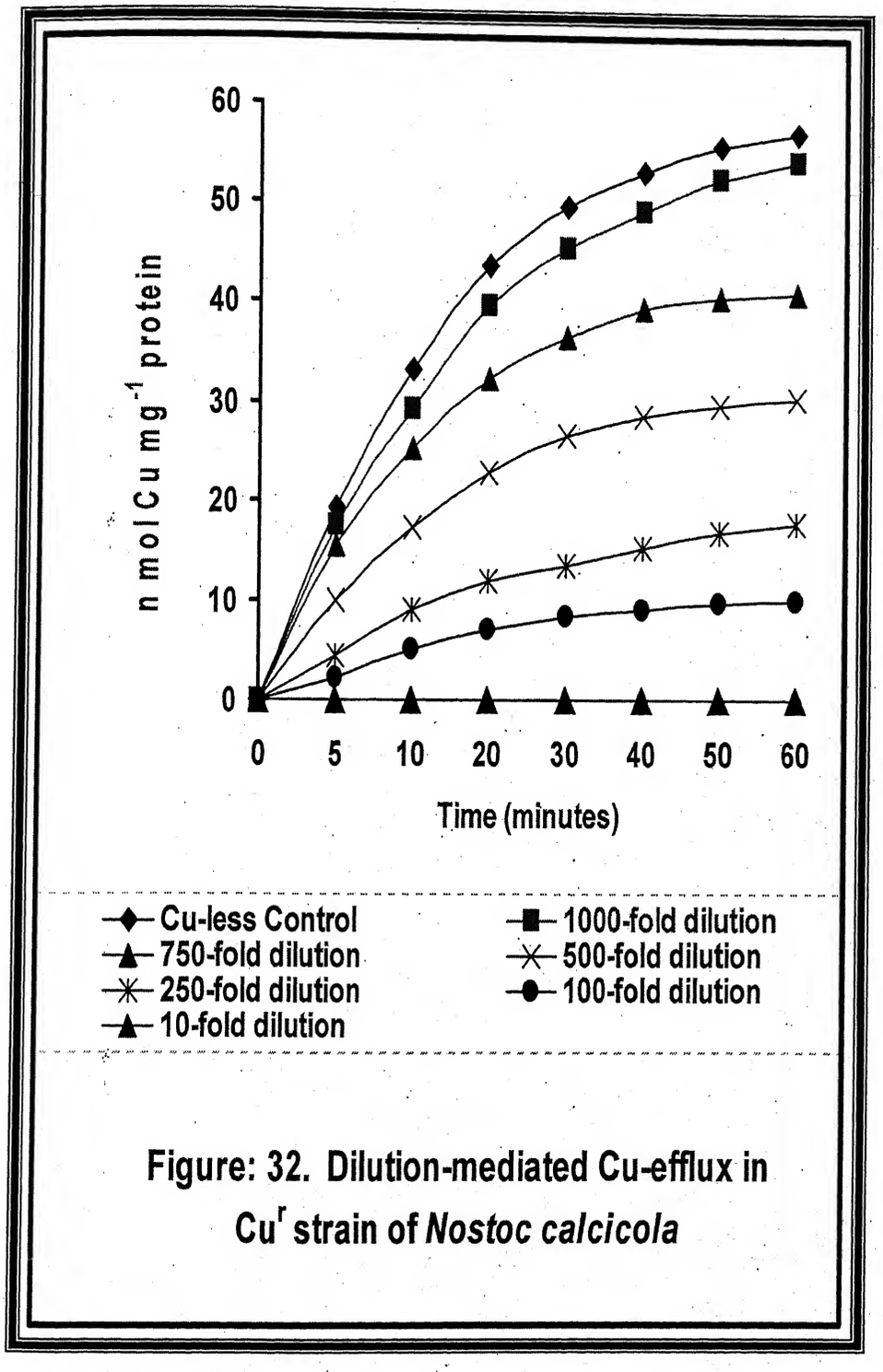

Figure: 32. Dilution-mediated Cu-efflux in Cu^r strain of *Nostoc calcicola*

5.7.4. THRESHOLD Cu^{2+} CONCENTRATION FOR Cu^{2+} EFFLUX:-

Figure: 33 compares the data on Cu^{2+} efflux *vs.* intracellular Cu-level in Cu^r strain of *Nostoc calcicola*. As the data shows there was no Cu-efflux up to the cellular buildup of 19.8 n mol Cu^{2+} mg^{-1} protein. The efflux started very slowly up to certain limit but the efflux was found to be maximum (57.12 n mol Cu^{2+} mg^{-1} protein) at 60 min whereas the cellular Cu^{2+} level was 9.2 n mol Cu^{2+} mg^{-1} protein.

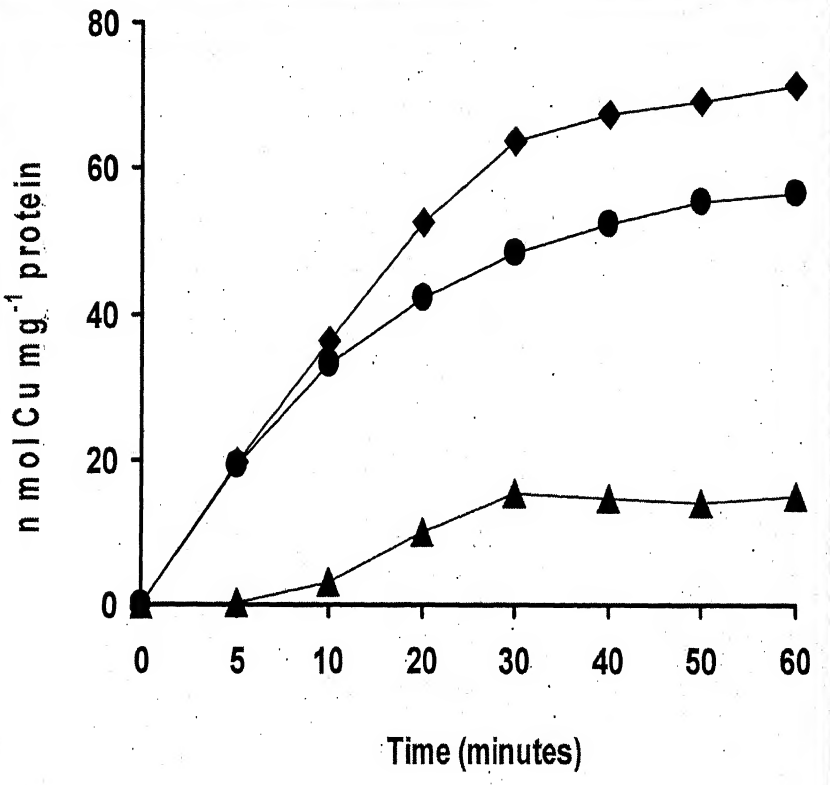

Figure: 33. Correlation between Cu-efflux and remaining cellular Cu level in Cu^r strain of *Nostoc calcicola*

CHAPTER – 6.

DISCUSSION

CHAPTER – 6.
DISCUSSION

6.1. COPPER UPTAKE:-

Metal accumulation by microbes generally comprises of two distinct phases:-

(a) Rapid binding of cations to the negatively charge groups on the cell surface and

(b) Subsequent metabolism-dependent intracellular uptake.

Such conclusions are based on metal uptake studies in yeast (Norris and Kelly, 1977), green algae (Gipps and Coller, 1980; Wilknson *et al.,* 1989), unicellular algae (Khummongkol *et al.,* 1982) and several cyanobacteria including *Choococcus paris* (Les and Walker, 1984), *Nostoc muscorum* (Schecher and Driscoll, 1985), *Anacystis nidulands* (Singh and Yadava, 1985), *Anabaena cylindrica* (Pettersson *et al.,* 1986; Campbell and Smith, 1986) and *Nostoc calcicola* (Singh *et al.,* 1989; Pandey *et al.*, 1993).

The pattern of Cu uptake in *Nostoc calcicola* showing concentration-dependence (in range of 10-60μM Cu; **Figure: 1**) is analogous to those reported earlier for Zn, Cd and Cu uptake in *Anacystis nidulands* (Shehata and Whitton, 1982; Singh and Yadava, 1985; Singh, D.P., 1985), Zn uptake in *Stigeoclonium* and *Cladophora* (Kelly and Whitton, 1989; Whitton *et al.,* 1989), Hg^{2+}uptake in *Nostoc*

calcicola (Pandey *et al.*, 1993) and Ca^{2+}uptake in *Nostoc calcicola* (Pandey *et al.*, 1996). The aim behind shifting to immobilized *Nostoc calcicola* cells was offer a comparison of the metal uptake events with those of free cells just described. The enhanced metal buildup in immobilized cells has proved the superiority of immobilization **(Figure: 2).** The immobilized cyanobacterial cells also took up Cu in a concentration-dependent manner as observed for free cells. Such obvious differences in Cu intake are also seen if one compares Cu^{2+} uptake rate of both free and immobilized cells. These observations eventually encouraged me to look for the improved functional longitivity of immobilized *Nostoc calcicola* cells as noted in others with reference to higher yield of nitrogen fixation (Vincenzini *et al.*, 1981), H_2-evolution, ammonia production (Brouers and Hall, 1985, 1986) and higher photosynthetic activity (Lambert *et al.*, 1979).

Nostoc calcicola cells under both the states, exhibited concentration-dependent metal uptake with higher metal uptake in immobilized cells. Such changes indicate that the enhanced accumulation of Cu in immobilized cells could be due to the improved rates of Cu intake. However, the nature of Cu uptake kinetics invariably remained biphasic for either free or immobilized cells. In similar comparisons the immobilized cells of *Chlorella emersonii* accumulated more Hg than free cells (Wilkinson *et al.*, 1989). These investigators also observed that in long term experiments, the immobilized cells accumulated significantly more Hg than free cells maintaining an overall difference of 30 events for 12 days.

In the majority of cases, the photoautotroph including cyanobacteria drive their energy-dependent processes at the expense of

light-generated ATP, and organisms exposed to dark conditions fail to maintain the optimum level of metabolic reactions or may have it at a fairly low level. Cell permeabilization by mild treatment with organics like toluene or acetone has been used to account for the active and passive uptake of ions in many cases. Gadd and Griffith (1978) reported that the level of metal intake by passive mechanism and the metal bound on the algal cell surface remains reasonably low compared to the amount taken in though the metabolic or energy-dependent reactions. While majority of metal intake events reported in algae are in favour of their dependence on cellular energy (Singh and Yadava, 1985; Campbell and Smith, 1986), there are reports that Al uptake in *Anabaena cylindrica* (Pattersson *et al.,* 1986) and Zn uptake in *Cldophora glomerata* (Vymazal, 1987) is not energy dependent. Since the present experiments with *Nostoc calcicola* were performed under light conditions, the photosynthetically generated energy remains common to the normal and permeabilized cells as well, and any difference in the respective Cu intake values would be attributed to the integrity of the cell membrane. Such observations would also allow one to account for the active and passive uptake. The lower curve in **Figures: 3 and 4,** is typical of the normal pattern as observed for non-toluene treated, control *Nostoc calcicola* with the uptake rate of 5.43 n mol Cu mg^{-1} protein min^{-1} (up to 10 min) with a saturation initiating after 50 min in the Cu uptake medium. The permeabilized cells on the other hand not only maintained higher initial Cu intake rate (8.74 n mol Cu mg^{-1} protein min^{-1}, 10 min), but also a higher profile of Cu uptake with the overall 1.52-fold difference. Also, the trend of Cu intake in the latter case, attainted saturation beyond 20 min. Assuming that the permeabilized cells are showing a combination of active and passive uptake, the net value of passive uptake comes to 54 n mol Cu mg^{-1}

protein. The permeabilization of cells prior to immobilization also resulted in the corresponding increase in Cu uptake, suggesting that the gel entrapped permeaplasts also behave like free cells as far as the active and passive transport of ions is concerned (comparison of **Figures: 3. and 4).**

6.2. REGULATORY MECHANISM :-

Energy generated by various metabolic pathways is conserved in the form of ATP, which acts as a generator of membrane potential across the membrane and can also be used to bring about concentration work; i.e., the work required transporting an ion or a molecule across the membrane. Cu uptake by *Nostoc calcicola* cells under light and dark conditions showed a wide difference that light-grown cells maintained a 14-fold difference in free cells and 1.5-fold in immobilized cells over the dark incubated cells in each state, suggesting for the photosynthetically generated energy-dependent Cu uptake **(Figures: 5 and 6).** Similar results were also reported for Ni uptake in *Anabaena cylindrica* (Campbell and Smith, 1986); Hg^{2+} uptake in *Nostoc calcicola* (Pandey *et al.,* 1993). Contrary to such observation observations, similar initial Cd uptake in light and dark grown cells of *Anabaena cylindrica* (Pettersson *et al.,* 1986), Zn accumulated by *Cladophora* (Vymazal, 1987) were also reported which disfavour the energy and metabolism dependent cellular transport of ions. The lesser reduction in Cu uptake by dark-grown immobilized cells in comparison to free cells may be due to the higher reserve of photosynthetically generated energy / reductant in the former as suggested by Affolter and Hall (1986) and Kerby *et al.,* (1986).

Although a different situation has been encountered with the reports of Papagaeorgiou *et al.* (1988) that immobilized cells of *Anabaena nidulans* have photosynthetic electron transport at 28% of efficiency of free cells and in other words, this 72% inhibition was attributed to photonic as well as diffusional limitations. Also a slight improvement in efficiency to 30% of the free cells at saturating light conditions (800 Watt m^{-2}) leaves diffusion barrier alone responsible for the overall 70% inhibition of photosynthetic electron transport. Subsequent to the exogenous supply of ATP (10μM) to "dark" cells in free state, there was a favorable response in terms of improvement in metal uptake by a factor of 10 over the non-supplemented free cells. The immobilized cells, however, did not show much reduction in Cu uptake even after 72 h dark incubation. Exogenously added ATP could not raise Cu uptake in such "dark" cells, thus suggesting that immobilization cells were still able to sustain ATP reserve optimum to drive the active transport of ions including Cu. Similarly, Potts and Morrisson (1986) reported that immobilization cells of *Nostoc commune* maintain their ATP pool size when undergoing various shifts in metabolism.

Nostoc calcicola under both the conditions (free and immobilized) exhibited time dependent metal uptake with the superiority of immobilized cells over free cells **(Figure: 7).** This indicated the enhanced accumulation of Cu in immobilized cells. In a similar comparison, the immobilized cells of *Chlorella emeresonii* accumulated more Hg^{2+} than free cells (Wilkinson *et al.,* 1989). These investigators also observed that in long term experiments, the immobilized cells accumulated significantly more Hg^{2+} than free cells maintaining an overall difference of 30% even for 12 days.

Data in **Figures: 8 and 9** incorporate the respective contribution of selected metabolic inhibitors / uncouplers towards Cu-uptake in free and immobilized *Nostoc calcicola* cells.

Thiols (mono and di) are known to reduce metal toxicity Agrawal and Kumar, 1977, Rai and Raizada, 1985, 1987) as well as uptake in case of Cd (Singh and Yadava, 1985) and Cu (Verma and Singh, 1990). Non-inhibitory concentration of mercaptoethanol (30μM) brought about an almost 25% inhibition in metal uptake over the control cells. The effectiveness of mercaptoethanol was also evident from very beginning (5 min).

Azide (10μM), the well known inhibitor of respiratory electron transport and uncouple of oxidative photophosphorylation (Hewitt and Nicholas, 1963), is even a substrate for *nitrogenase* (Rubinson *et al.*, 1985) inhibited Cu-uptake more than 50% (**Figure: 8**; 3rd curve from top) since the treatment medium did not contain any combined nitrogen. It is presumed that azide could be used as a *nitrogenase* substrate in a major way while lesser amount could be available inhibitor / uncoupler of processes mentioned above.

The 4th curve with a strong inhibition of Cu-uptake by DCCD (10μM) even within first 10 min was due to alteration in the energy transfer processes *via* inhibition of *ATP synthase* dependent ATP synthesis (Peschek *et al.*, 1979).

The strongest inhibition (100%) by pCMB (1μM) with in Cu-uptake can be explained on the basis of the interaction of pCMB with –SH groups present on the cell membrane (Brachet, 1975) are always

signifying the integrity of the membrane integrity of the membrane to maintain active ion-transport. The cell membrane, as well as the –SH groups containing enzyme receptors might be effected as pCMB in term through a damaged plasma-membrane resulting in the complete abolition of metal uptake in both the states.

The pattern of Cu uptake by ageing free *Nostoc calcicola* cells remained maximum within 6 and 12 days, thus suggesting that the active import of the metal is characteristic of exponentially growing cells **(Figure: 10).** A sharp contrast representing Cu intake by immobilized cells, certainly speaks of their longevity as sufficient Cu intake could be seen even in one month old beads. Lambert *et al.,* (1979) observed higher O_2 evolution in immobilized *Anabaena cylindrica* cells even up to 50 days in comparison to free cells that could retain it only for 20 days. Other sustained metabolic event includes H_2 evolution lasting several weeks in immobilized *Chomatium* cells (Ikemoto and Mitsui, 1983) and *Rhodopseudomonas* (Mitsui *et al.,* 1985). Cell ageing was also delayed in immobilized *Vicia faba* in relation to parameters like O_2 evolution, C-fixation and pigment degradation (Schnabl *et al.,* 1983). Long term Cu uptake experiments involving free and immobilized *Nostoc calcicola* cells have clearly shown the sustained metabolic activity in the latter case. Free cells showed Cu uptake (96.89 n mol Cu mg^{-1} protein) up to a maximum of 12 days and the decline thereafter, was indicative of cell ageing. Immobilized cells on the other hand, attained a maximum of 300.82 n mol Cu mg^{-1} protein on the 12^{th} day and it was only thereafter that cells in such a state showed decline in Cu intake. Immobilized technique has been successful in achieving successful in achieving sustained hydrogen production in marine and other photosynthetic bacteria

(Matsunaga and Mitsui, 1982; Singh *et al.,* 1990) and cyanobacteria (Mitsui *et al.,* 1985). Similarly, high yields of H_2 production by immobilized *Chomatium* led to the suggestion that energy production and the removal of sulphide from polluted water-bodies could be simultaneously achieved following immobilization (Ikemoto and Mitsui, 1984). The apparent superiority of immobilized cells in long term Cu uptake experiments thus suggests that that such a system could be successfully applied to remove heavy metals from the polluted waters though repeated cycles and with no loss of cells during transfer; a problem that generally emerges during handling and concentration of free cells before shifting to the new growth medium.

6.3. PHOSPHATE UPTAKE AND *ATPase*:-

The cyanobacteria have, like many other algae, the capacity to incorporate and store phosphorus-phosphate from the external medium with a few exceptions (Batterton and Van Baalen, 1968). The majority of reports are in favour of the energy-dependent uptake of phosphorus-phosphate and its accumulation in cyanobacteria (Falkener *et al.,* 1980). Light plays an important role in regulation of its uptake in cyanobacteria (Batterton and Van Baalen, 1968; Healey, 1973; Grillo and Gibson, 1979). The continuously increasing rate of phosphate uptake in the phosphate-starved (12 h) cells even up to 5 h indicates the possible operation of enzyme *polyphosphate synthetase* **(Figure: 11)** and the increased activity of this enzyme has been taken as the biochemical basis for the formation of polyphosphate bodies during growth of phosphate-limited cyanobacteria in phosphate-excess medium (Grillo and Gibson, 1979). Phosphate uptake in Cu-less free

cells increased linearity up to 4 h with saturation at 5 h attaining a maximum of 0.78μ mol PO_4^{3-} μg^{-1} protein. Cu loading of such cultures caused a concentration-dependent inhibition of phosphate uptake with 50% decline by 20μM Cu. The 60μM Cu concentration causing 86% inhibition of phosphate uptake, suggested the hypersensitivity of the process in comparison to any other process studied so far. Phosphate uptake in immobilized *Nostoc calcicola* cells was slightly higher over free cells attaining maximum of 1.12μ mol PO_4^{3-} μg^{-1} protein after 5 h. For such cells there was a concentration-dependent inhibition of of uptake between 20-80μM Cu **(Figure: 12).** Immobilization as in previous cases also resulted in the resistance of cells as 60μM Cu inhibiting 86% of phosphate uptake in free cells, caused 52% less inhibition of uptake. Also, the maximum Cu concentration for immobilized cells (80μM) allowed 12.25% phosphate uptake.

There are reports covering heavy metal inhibition of phosphorus-phosphate uptake in cyanobacteria (Singh and Yadava, 1984) and phosphate uptake and photosynthesis of planktonic communities in selected Precambrian shield lakes (Nalewajko and Paul, 1985). The nature of Cu inhibition in free as well as immobilized *Nostoc calcicola* cells was non-competitive thus ruling out the possibility of a direct encounter between Cu and phosphate at the cell exterior. Pettersson *et al.,* (1988), observed that Al, severly affected growth of the cyanobacterium *Anabaena cylindrica* and induced symptoms of phosphorus-starvation. However, these investigators could not observe Al-inhibition of phosphate uptake, and the rapid accumulation of polyphosphate granules in cells exposed to Al in such cases, also established that the cation did not disturb phosphate incorporation although it lowered the activity of enzyme acid

phosphatase along with the mobilization of polyphosphate. They conclude that Al acts on the intracellular metabolism of phosphate, which eventually leads to phosphorus-starvation rather than on its uptake in *Anabaena cylindrica*. Higher phosphate uptake by immobilized *Nostoc calcicola* cells in the present case may be due to their higher energy state in comparison to free cells. There are similar observations by Chevalier and de la Noue (1985) on immobilized *Scenedesmus quaricaudata, S. obliquus* and *S. acutus* that quite efficiently removed nitrogen and phosphorus from urban secondary effluents. However, such was not the case with immobilized *Chlorella* as the rate of phosphoprus uptake remained much lower than those of non-immobilized cells (Robinson *et al.,* 1988, 1989).

There is evident for the presence of two types of *ATPases* in cyanobacteria: Ca^{2+}-dependent *ATPase* is the coupling factor of the thylakoid mediating photosynthetic production of ATP. Mg^{2+}-dependent *ATPase* predominately located in plasmamembrane and supposed to play a vital role in cation transport. Lockau and Pfeffer, (1982) observed sensitivity of these enzymes towards vanadate. The immobilized cells as control in the present investigation, showed nearly 40% less *in vivo* activity of Mg^{2+}-dependent *ATPase* in comparison to free cells. Similarly, there was more than 20% drop in Mg^{2+}-dependent *ATPase* activity in such cells compared to free cells (comparison of **Figures: 13, 14, 15 & 16).** Such observations suggest that the immobilized cells maintain sufficient ATP pool as applicable to *Nostoc commune* (Potts and Morrison, 1986). It is evident from the Cu exposure of both the *ATPases,* that the metal stimulate enzyme activity in free as well as immobilized cells although high Cu concentration (40-80μM) could be used for the purpose. Similar stimulation of only

Mg^{2+}-dependent *ATPase* by Cd or Ni (0.5 mol m^{-3}) has been reported in rice plant by Ros *et al.,* (1990). Similar enzyme activities were also recorded on the *in vitro* enzyme activity in both the cases. **(Figures: 17, 18, 19 & 20).**

Stimulations in membrane *ATPase* by xenobiotics has been attributed to alterations in sterol and phosholipid composition of the plasmamembrane (Cooke *et al.,* 1989). The present observations clearly establish that Cu affect both the enzymes (Ca^{2+}- and Mg^{2+}-dependent *ATPases*) or in other words, results in more ATP hydrolysis in either free or immobilized state of *Nostoc calcicola* cells. Inhibition by Cu of both the enzymes that elevated concentrations can be attributed to the Cu-effect on plasmamembranes of the cyanobacterium although no experiments have been done to compare the plasmamembrane integrity under metal stress.

6.4. *NITROGENASE:-*

Nitrogense is the key enzyme in biological nitrogen fixation by photoautotrophic microorganism including cyanobacteria (Stewart and Lex, 1970; Stewart, 1973, 1980; Stewart *et al.,* 1975). The metal-less free cells showed increasing trends in the enzyme activity attaining a maximum of 6.12 n mol C_2H_4 produced mg^{-1} protein min^{-1} **(Figure: 21).** The present observation on deleterious effect of Cu on *nitrogenase* activity reveal that the process was sensitive to all the metal concentrations used (60-500μM). The Cu concentration (60μM) brought about a 26% decline in *nitrogenase* activity (at 3 h) although lowering could be seen even after 30 min of metal exposure of cells.

The extent of inhibition of *nitrogenase* activity was concentration-dependent (60-500μM). It may also be noted that the algal cells retained at least some level of *nitrogenase* activity (0.72 n mol C_2H_4 produced mg^{-1} protein min^{-1}; 3 h) at the highest metal concentration used (500μM Cu).

Immobilization resulted in the resistance of cells against Cu inhibition because 60μM Cu causing 27% decline in *nitrogenase* activity of free cells, could do so only by 7.15%, and the Cu-resistance of immobilized cells was also reflected by a comparison of 50% inhibitory concentration as it came to 500μM Cu compared to 100μM Cu for free cells just described **(Figure: 22).** The superiority of immobilized cells over their free cell counterpart could also be seen as *nitrogenase* activity is less inhibited in immobilized cells against Cu^{2+}.

The present observations on *nitrogenase* inhibition by Cu are analogous to the previous reports on inhibition of *nitrogenase* by heavy metals like Cu, Cd, Hg, Ni, Cr, Ag, Pb, Zn As or Al (Horne and Goldman, 1974; Henriksson and DaSilva, 1978; Stratton and Corke, 1979; Stratton *et al.,* 1979; Petterssonhn *et al.,* 1985; Rai and Raizada, 1985, 1986; Raizada and Rai, 1985; Dubey and Rai, 1987; Singh *et al.,* 1987). A decline in nitrogen fixation by the Cu treated *Aphanizomenon flos-aquae* cells have been attributed to: (a) the breakdown of photosynthesis in vegetative cells that ultimately channelize energy to heterocyst (Wurtsbaugh and Horne, 1982; Scherer *et at.,* 1988) and (b) irreversible inactivation of *nitrogenase* because of insufficient supply of energy to remove oxygen within heterocyst (Foy and Cox, 1967; Murray and Horne, 1980). It may also be mentioned that *Nostoc calcicola* cells while taking up Cu concentration (60μM),

concomitantly damage their various physiological processes, nitrogen-fixation being one of them as the activity went down by 33% within 1 h. The increased resistance of immobilized cells speaks of their superiority over free cells. The enhanced *nitrogenase* activity in immobilized control cells is in line with other reports (Musgrave *et al.*, 1982; Brouers and Hall, 1986; Kerby *et al.*, 1986; Shi *et al.*, 1987; Karpunina *et al.*, 1989). Also, the enhanced heterocyst frequency of immobilized *Nostoc calcicola* cells over free cells; is in agreement with the reports of Musgrave *et al.*, (1982) for *Anabaena* sp. with the heterocyst frequency up to 9.8 over 7.5% in free cells. The endophytic *Anabaena azolla* could even double its heterocyst frequency following immobilization (Hall *et al.*, 1985; Shi *et al.*, 1987).

GLUTAMINE SYNTHETASE (TRANSFERASE):-

Glutamine synthetase (L-glutamate; *ammonia ligase,* adenosine-5'-diphosphate forming) is the main enzyme involved in cyanobacterial NH_4^+ assimilation (Stewart, 1980; Chapman and Meeks, 1983) and may be regarded as the first step in biosynthesis of vital metabolic compounds. *Glutamine synthetase (transferase)* activity in N_2 grown, *Nostoc calcicola* free cells (basal level 1.25µ mol γ-glutamylhydroxamate mg^{-1} protein min^{-1}) attained a maximum of 2.4µ mol γ-glutamylhydroxamate mg^{-1} protein min^{-1} within 3 h of photoincubation **(Figure: 23).** Based on comparisons made for *nitrogenase* inhibition just before, the *glutamine synthetase* activity was slightly more sensitive to Cu (60µM) as reflected by a 24% inhibition within 1 h of Cu exposure. Similarly, immobilized cells showed 05% inhibition of enzyme activity within 1 h exposure to Cu

concentration (60μM; **Figure: 24).** The sensitivity of *glutamine synthetase* to increasing Cu concentrations for both the conditions of cells of *Nostoc calcicola,* is quite comparable to the inhibition of *nitrogenase* **(Figures: 23 and 24).** Since such preliminary experiments fail to recognize the very sites of Cu action, it can be suggested that the observed inhibition could possibly arise from the disrupt energy supply resulting from the Cu-membrane interaction and / or such ions could occupy the action site first then move to the regulatory site(s) of enzyme following saturation. Compared to *nitrogenase*, *glutamine synthetase* remains little explored in terms of the enzyme *vs.* heavy metal interaction as reported by Singh *et al.,* (1987) on *glutamine synthetase* (*transferase*) sensitivity towards Hg^{2+} and Cu^{+2} and that of Ip *et al.,* (1983) on the binding of cations like Hg, Cd, Mn, Co, Ni, Cu, Zn, Ca, Ba, Fe, Sr, or Sn with the regulating sites of GS (biosynthetic) and in turn, impose regulations on the enzyme activity.

6.5. $^{14}CO_2$ INCORPORATION:-

As cyanobacteria depend largely on photosynthesis for the generation of ATP and reductants, the present study was an attempt to ascertain the nature of Cu action with respect to such aspect in *Nostoc calcicola* cells involving experiments on $^{14}CO_2$-incorporation against Cu ions ranging from 30-60μM for free and immobilized *Nostoc calcicola* cells **(Figures: 25 and 26).** The free cells as control showed 13,580 CPM mg^{-1} protein $^{14}CO_2$-uptake at 3 h and such cells dosed with Cu concentration (60μM), displayed a 30% reduction within 1 h that reached 100% at 3 h. The immobilized control cells as in other comparisons, proved more efficient also in $^{14}CO_2$ uptake with an

overall 1.8-fold increase over free cells. The Cu concentration (60μM) could reduce $^{14}CO_2$-uptake by only 18% during 1 h or a maxim of 36% at 3 h exposure. The inhibitory effects of Cu on $^{14}CO_2$-incorporation in *Nostoc calcicola* are in line with the report of Rai and Raizada (1985) on Ag and Ni inhibition of carbon-fixation in *Nostoc muscorum* and that of singh and singh (1987) on Hg in *Nostoc calcicola.* Takamura *et al.,*(1989) have taken carbon-fixation as a criterion for comparing heavy metal sensitivity of cyanobacterial strains as well as others from different algal groups and observed that cyanobacteria were sensitive to Cu, Cd or Zn wether these originated from the polluted sites or not. The concentration of Cu that caused 50% inhibition of photosynthesis was 3.5μM for *Phormidium ramosum* and 0.01μM for *Chamaesiphon polymorphus.* The physiological basis of Cu inhibition of photosynthesis has been in terms of inhibition of dark reaction in *Chlorella vulgaris* (Greenfield, 1942, retardation of ferredoxin-dependent reaction (Babich and Stotzky, 1978; Shioi *et al.,* 1978; De Filippis *et al.,* 1981), metal action on photochemical processes generating reductants from Calvin cycle (Cedano-Maldonado *et al.,* 1972; Singh and Singh, 1987; Scherer *et al.,* 1988).

Since Cu inhibited $^{14}CO_2$-assimilation in free as well as immobiliozed cells and the degree of inhibition was less marked for gel entrapped cells, the observed tolerance of immobilized cells against Cu inhibition of $^{14}CO_2$-incorporation may be suggested to arise from the following reasons reported in other organism: - (a) higher photosynthetic O_2 evolution in the immobilized *Botrycoccus braunii* (Bailliez *et al.,* 1986) and *Anabaena* sp. (Lambert *et al.,* 1979; Affolter and Hall 1986; Kerby *et al.,* 1986), and (c) the excretion of secondary metabolites in the ambient medium by plant cells that could bind the

metal though chelation (Brodelius and Mosbach, 1982; Bucke 1983; Brodelius 1984, 1985) or (d) the maintainance of sufficient cellular ATP pool in *Nostoc commune* to drive the various physiological reactions (Potts and Morrison, 1986).

6.6. COPPER RESISANT / TOLERANT STRAIN:-

In general, the acquisition of heavy metal resistance in algae has been associated with the lowered uptake of the cations in the resistant strain as evident from observations in Cd-tolerant *Anacystis nidulans* (Singh and Yadava, 1986), Cu-tolerant *Scenedesmus* (Mierle and Stokes, 1974) including the Cd-resistant bacterium *Staphylococcus aureus* (Tynecka *et al.,* 1981.a). There are also reports that Zn uptake in Zn-tolerant strain of *Anacyatis nidulans* was at par with the sensitive strain (Shehata and Whitton, 1982). The investigations have also revealed the operation of the two alternative mechanisms in a Ni-tolerant *Synechococcus* ATCC 17146 strain:-

(a) The mutant in the first category synthesized intracellular synthesized intracellular polymers to complex with the imported Ni, and

(b) Those that did not permit intracellular import of the cation possibly due to extrusion of low molecular weight complexans to engage the cations extra-cellularly (Gleason and Wood, 1988).

Also, in the Cd-resistant yeast cells, the characteristic of decreased ion uptake was thought to be the only resistance mechanism

operative (Tohoyama and Murayama, 1977). For *Nostoc calcola* cells in the present case, the Cu^r strain showed a slightly lower pace for growth compared to its parent in the normal growth medium lacking metal although it tolerated 70μM Cu compared to Cu^s strain showing lethality at 5μM **(Figure: 27).**

Experiments covering Cu uptake in the Cu^s and Cu^r strains revealed that the latter belonged to "less uptake" category as described before. The overall pattern of Cu-uptake event in Cu^r strain was characterized by lowered uptake rate (1.2 n mol Cu mg^{-1} protein min^{-1}) as the 26% reduction in the total Cu-intake (compared at 60 min; **Figure: 28).**

Experiments directed towards knowing the possibility of Cu efflux in Cu^s and Cu^r strains of *Nostoc calcicola* have revealed that the latter was characterized by time-dependent (0-60 min) Cu-efflux compared to almost no cation efflux in the Cu^s strain **(Figure:29).**

Such observations are supported by the fact that Cu^r strains of *Nostoc calcicola* could account for more than 80% Cu-efflux (57.12 n mol Cu mg^{-1} protein) over the same cation taken in during the same period (72.15 n mol Cu mg^{-1} protein; 60 min). Also, the Cu-uptake rate as calculated at 20 min (2.6 n mol Cu mg^{-1} protein min^{-1}), differed by a narrow margin from that of its efflux (2.13 n mol Cu mg^{-1} protein min^{-1}) in case of Cu^r strain incubated in a normal strength growth medium. Almost a straight line running parallel to abscissa indicates that the parent cellular buildup for Cu-uptake (96.89 n mol Cu mg^{-1} protein), were not inducible in term of Cu-efflux when exposed to a fresh Cu-less medium. Verma and Singh (1991) also observed similar trend in

Cu-resistant *Nostoc calcicola* strain (60μM) amounting to almost 90% efflux within 1 h of incubation of cells in a Cu-free medium.

6.7. FACTORS REGULATING COPPER EFFLUX IN Cu^r STRAIN OF *Nostoc calcicola* :-

The present experiments with Cu^r strain of *Nostoc calcicola* also suggest that Cu efflux depended to a major extent on light generated ATP as only 1 h dark-exposure of such cells, brought about 95% inhibition of Cu efflux; exogenous supply of ATP (10μM) to such cells, could restore only 50% of Cu efflux **(Figure:30).**

Since the addition of similar amount of ATP to "light" cells of Cu^r strain did not improve Cu efflux over the light (-ATP) sets. It is suggested that photosynthetically generated energy was sufficient enough to drive Cu efflux. In a similar study with bacterial system, Tynecka *et al.,* (1981. b), observed that plasmid dependent cadmium resistance in *Staphylococcus aureus* was due to operation of an energy dependent efflux system that in turn prevented intracellular Cd buildup. The possible mechanism involved, was the energisation of efflux system by proton circulation across the membrane, and this probably mediated the exchange of cations like Cd^{2+} for protons. These investigators also observed that the cation efflux was dependent on metabolic energy like cation influx as reported in case of *Nostoc calcicola* (Verma and Singh, 1990). Similar efflux mechanism is also known to operate for tetracycline or arsenate in *Escherichia coli* (Ball *et al.,* 1980; Silver and Keach, 1982).

Figures: 31 incorporates the respective contribution of selected metabolic inhibitors / uncouplers towards Cu-efflux in Cu^r strain of *Nostoc calcicola.* Results were fewer values due to less Cu-uptake in Cu^r strain.

Thiols (mono and di) are known to reduce metal toxicity Agrawal and Kumar, 1977, Rai and Raizada, 1985, 1987) as well as efflux in case of Cd (Singh and Yadava, 1985) and Cu (Verma and Singh, 1990). Non-inhibitory concentration of mercaptoethanol (30μM) brought about an almost 25% inhibition in metal-efflux. The effectiveness of mercaptoethanol was also evident from very beginning (5 min).

Azide (10μM), the well known inhibitor of respiratory electron transport and uncouple of oxidative photophosphorylation (Hewitt and Nicholas, 1963), is even a substrate for *nitrogenase* (Rubinson *et al.,* 1985) inhibited Cu-efflux more than 50% (**Figure: 31**; 3rd curve from top) since the treatment medium did not contain any combined nitrogen. It is presumed that azide could be used as a *nitrogenase* substrate in a major way while lesser amount could be available inhibitor / uncoupler of processes mentioned above.

The 4th curve with a strong inhibition of Cu-efflux by DCCD (10μM) even within first 10 min was due to alteration in the energy transfer processes *via* inhibition of *ATP synthase* dependent ATP synthesis (Peschek *et al.,* 1979).

The strongest inhibition (100%) by pCMB (1μM) with in Cu efflux can be explained on the basis of the interaction of pCMB with –

SH groups present on the cell membrane (Brachet, 1975) are always signifying the integrity of the membrane integrity of the membrane to maintain active ion-transport. The cell membrane, as well as the –SH groups containing enzyme receptors might be effected as pCMB in term through a damaged plasma-membrane resulting in the complete abolition of Cu-efflux.

Since there was significant Cu efflux from Cu^r strain cells suspended in normal strength growth medium, it was quite tempting to go for the possible role of dilutions of the growth medium, thus amounting to varying Cu strength. The observations in this direction demonstrate the vital role of Cu amount in the ambient medium **(Figure: 32).** The data incorporated therein are based on the time course (0-60 min) and degree of dilutions (10-1000 fold) as the variables. It may be indicated that 10-fold diluted medium contained 6μM Cu, 1000-fold dilution was left with only 0.06μM Cu concentrations adjusted accordingly.

It may be noted that Cu-efflux initiated for 100-fold dilution sets and not before. Also, there was no sharp difference in terms of Cu efflux rate (at 10 min) for either 250 or 500 fold dilutions, but the ultimate Cu output showed an increasing trend with the passage of time in the latter case. The efflux rate (2.42 n mol Cu mg^{-1} protein min^{-1}; 10 min) and the total efflux of 41.01 n mol Cu mg^{-1} protein for 750-fold dilutions collectively indicated for an average of 26% Cu-recovery over the sets having 500-fold dilutions. The close and constant proximity between the rates of Cu efflux for 1000-fold diluted sets (0.93 n mol Cu mg^{-1} protein min^{-1}) and those of Cu-free medium (0.95 n mol Cu mg^{-1} protein; total Cu efflux 57.12 n mol Cu mg^{-1} protein) at 1 h suggests

that the highest dilution or the lowest amount of Cu (0.06μM) was regulatory to the efflux event. There is a single report of this type in the Cd-resistant *Staphylococcus aureus* as only a 10-fold dilution and not 1000-fold as in the present case, could cause complete cation effluxd (Tynecka *et al.*, 1981). Secondly, the shorter time (10 min) consumed for complete Cd^{+2} efflux in *Staphylococcus aureus* compared to 1 h necessary for Cu efflux in Cu^r strain of *Nostoc calcicola* could be attributed to the higher rates of metabolism / growth in the former.

Figure: 33 compare the critical role of residual Cu level *vs.* Cu efflux. It is evident that the first 10 min incubation did not initiate Cu efflux possibly due to sufficient intracellular Cu. The significant Cu^{+2} efflux was recorded at 20 min. It is suggested that critical concentration of intracellular Cu has been attained and here onward, the efflux is having a negative correlation with that of intracellular Cu.

CHAPTER – 7.

CONCLUSION

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In short term uptake experiments in diazotrophic cyanobacterium *Nostoc calcicola* Breb; The Cu uptake pattern comprised of rapid binding of Cu^{+2} on the cell wall followed by metabolism-dependent intake. A tolerant strain of *Nostoc calcicola* has been isolated in the laboratory. This copper resistant strain may be used in bioleaching program. Cu-resistant (Cu^{r}) strain revealed its ability to grow at 70µM Cu. 5µM Cu being lethal for Cu-sensitive (Cu^{s}) strains. The overall pattern indicated the acquisition of metal tolerance in the former. This research work is helpful in the removal of excess Cu from polluted environment without any measurable loss, as the immobilized cells may be used in the treatment of Cu pollution.

It is presumed that the research wok will help the fellow researchers for their future plans in the research aspects of bioremediation and for anyone who likely to develop the interest to remove metals such as arsenic, cadmium, cobalt, lead, magnesium, mercury, nickel, zinc, etc. from the polluted environment using cyanobacteria.

CHAPTER – 8.

SUMMARY

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SUMMARY

The sensitivity of selected physiological and biochemical studies were made on free and immobilized cultures of diazotophic cyanobacterium *Nostoc calcicola* Breb, exposed to copper. The results obtained are summarized as:-

(1). Cu-uptake pattern in both free and immobilized cells was typically biphasic, comprising of (a) rapid binding of Cu on the cell wall followed by (b) the subsequent metabolism-dependent uptake.

Cu uptake in free cells at 60μM Cu^{+2} with a metal buildup of 96.89 n mol Cu mg^{-1} protein min^{-1}; in comparison, immobilized cells showed 3-fold (300.82 n mol Cu mg^{-1} protein) at 60μM Cu^{+2}.

(2). Free cells exposed to dark (72 h), showed an abrupt decline in Cu uptake (7.16 n mol Cu mg^{-1} protein) amounting to more than 9-fold difference from the light-grown control cells (96.89 n mol mg^{-1} protein). Such dark cells when supplied with exogenous ATP (10μM), showed enhanced Cu uptake by a factor of 10. The incomplete recovery in such a case could be attributed to the short supply of reductants in cells exposed to dark. The dark-incubated immobilized cells on the other hand, reflected only a 1.34 fold lowering in Cu uptake (200.15 n mol Cu mg^{-1} protein). Such comparisons clearly indicate that immobilized calls have sufficient energy in reserve even if subjected to

non-photosynthetic conditions (72 h). The exogenously supplied ATP (10μM) to "dark" beads also; could not raise the Cu uptake to the level of "light" beads.

(3). Immobilized cells were characterized by a faster rate of phosphate uptake than the free cells. Cu inhibited phosphate uptake depending on the concentration as well as exposure time. Immobilization was non-competitive. Immobilization was associated with decrease *in vivo* activity of Ca^{+2} -dependent *ATPase* compared to a sharp decline approaching in case of Mg^{+2}-dependent *ATPase.* Lower Cu concentrations in contrast, were stimulatory to the activity of both the enzymes at least up to 1 h. The *in vivo* and *in vitro* activities of both the enzymes show similar pattern of Cu-inhibition, thereby ruling out any *in vivo* factor involved in regulating Cu toxicity to these enzymes in cyanobacteria.

(4). The higher *nitrogenase* activity in immobilized cells over the free cells has been attributed to (a) enhancement of heterocyst frequency, (b) creation of anaerobic condition, and (c) increase in the level of phototosynthetically generated ATP. Likewise, the immobilized cells were also characterized by higher GS activity in comparison to free cells.

(5). $^{14}CO_2$-uptake in immobilized cells was less sensitive to Cu compared to free living cells. 1 h Cu exposure of cells under both the state of cells was significant to know about the pattern of $^{14}CO_2$-uptake. The immobilized cells saturated for Cu uptake, could carry on $^{14}CO_2$-uptake more efficiently than the free cells.

(6). Characterization of Cu-resistant (Cu^r) strain revealed its ability to grow at 70μM Cu. The 5μM Cu is lethal concentration for Cu-sensitive (Cu^s) strains. The overall pattern indicated the acquisition of metal tolerance in the former. In Cu uptake comparisons, the Cu-resistant (Cu^r) strain showed only 26% less cellular metal buildup (72.15 n mol Cu mg^{-1} protein) at 1 h compared to Cu^s (96.89 n mol Cu mg^{-1} protein). Such a marginal reduction of Cu uptake in Cu-resistant (Cu^r) strain could not be taken as the sole mechanism of Cu tolerance as the same strain also showed significant Cu-efflux. Cu-efflux suggested being one of the mechanisms to develop heavy metal tolerance. In a time-course follow-up, Cu-resistant (Cu^r) strain showed almost 80% metal efflux (57.12 n mol Cu mg^{-1} protein) over the same period.

(7). Cu efflux in the Cu-resistant (Cu^r) strain depended to a major extent, on the light generated ATP as the "dark" cells brought about 95% inhibition. The exogenous supply of ATP to such cells could restore efflux by only 30% compared to no improvement in "light" cells thus suggesting that photosynthetically generated energy in the latter case was optimum to drive Cu-efflux. Cu-efflux in the Cu-resistant (Cu^r) strain also initiated only for 100-fold lower Cu concentration in the external medium (0.6μM Cu) and not before. The close and constant proximity between the Cu efflux rate for 1000-fold diluted sets and those of Cu free medium in the first 1 h suggested that higher dilution or the lower amounts of Cu (i.e.; 0.06μM in the present case) offered regulations for the efflux event.

CHAPTER – 9.

REFERENCES

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REFERENCES

Abou-Waly, H., Abou-Setta, M.M., Nigg, H.N. and Mallory, L.L. (1991) Growth response of fresh water algae *Anabaena flosaquae. Bull. Engviron. Contam. Toxicol.*, **46:** 223-229.

Adams, D.G. (1992) The effect of DL-azatryptophan on heterocyst development in cyanobacteria *Anabaena cylindrica. J. Gen. Microbiol.*, **138:** 355-362.

Adams, D.G. (2000) Heterocyst formation in cyanobacteria. *Curr. Microbiol.*, **3:** 618-624.

Affolter, D. and Hall, D.O. (1986) Long term stability of photosynthetic and electron transport in polyvinyl foam immobilized cyanobacteria. *Phytobiochem.* **11:** 193-201.

Agrawal, M.J. and Kumar, H.D. (1977) Cobalt toxicity and its possible mode of action in blue-green algae *Anacystis nidulans. Plant Physiol.*, **53:** 157-167.

Albertano, P., Pinto, G. and Taddel, R. (1978) Evaluation of toxic effects of heavy metals to unicellular algae: The influence of inoculum's concentration on the evaluation of toxicity. *Delpinoa.*, **20:** 75-86.

Ali, M.B., Tripathi, R.D., Rai, U.N., Pal, A. and Singh, S.P. (1999) Physico-chemical characteristics of pollution level of Lake Nainital, U.P., India. Role of macrophytes and phytoplankton in biomonitoring and phytoremediation of toxic metal ions. *Chemosphere.*, **139** (12): 2171-2182.

Allen, M.B. and Arnon, D.I. (1955) Studies on nitrogen fixing blue-green algae. Growth and nitrogen fixation by *Anabaena cylindrica. Plant Physiol.*, **30:** 366-374.

References

Allen, M.M. (1968) Ultra-structure of the cell wall and cell division of unicellular blue-green algae on plates., *J. Phycol.,* **4**: 1-4.

Allen, M.M. (1984) Cyanobacterial cellular inclusions. *Ann. Rev. Microbiol.,* **38:** 1-25.

Allen, M.M. and Smith, A.J. (1969) Nitrogen chlorosis in blue-green algae. *Arch. Microbiol.,* **69:** 114-120.

Alnutt, F.C.T. (1996) Cyanobacterial Biotechnology: Past, present and future. *J. Sci. Ind. Res.,* **55:** 693-714.

Amemiya, Y. and Nakayama, O. (1984) The chemical composition and metal adsorption capacity of the Sheath materials isolated from microcystis and cyanobacteria. *Curr. Microbiol.,* **45:** 187-193.

Anderson, D.M. and Morel, F.M.M. (1978) Copper sensitivity of *Gonynyaulaxn temerensis. Limnol. Oceanogr.,* **23:** 283-295.

Applebury, M.E., Johanson, B.P. and Coleman, J.E. (1970) Phosphate binding to *alkaline phosphatase. J. Biol. Chem.,* **245:** 4968-4975.

Armienta, M.A., Morton, O., Rodriguez, R., Cruz, O., Aguayo, A. and Ceniceros, N. (2001) Chromium in a tannery wastewater irrigation area. *Bull. Environ. Contam. and Toxicol.,* **66** (2): 189-195.

Asthana, R.K., Pandey, P.K. and Singh, S.P. (1990) Nickel regulation of photoautotrophy in a cyanobacterium. *Water, Air, Soil Pollut.,* **52 :** 263-276.

Asthana, R.K., Singh, S.P. and Singh, R.K. (1992) Nickel effects on phosphate uptake, *alkaline phosphatase* and *ATPase* of a Cyanobacterium. *Bull. Environ. Contam. Toxicol.,* **48:** 45-54.

Avery, S.V., Miller, M.E. and Gadd, G.M. (1991) Toxicity of organotins towards cyanobacterial photosynthesis and nitrogen fixation. *FEMS Microbiol. Lett.,* **84:** 205-210.

Azeez, P.A. and Banerjee, D.K. (1987) Influence of light on chlorophyll *a* content of blue-green algae treated with heavy metals. *Bull. Environ. Contam. Toxicol.,* **38:** 1062-1069.

References

Babich, H. and Stotzky, G. (1978) Effects of Cd on the biota: Influence of environmental factors. *Adv. App. Microbiol.,* **23:** 55-117.

Babich, H. and Stotzky, G. (1980) Environmental factors that influence the toxicity of heavy metals and gaseous pollutants to microorganisms. *Critical Rev. Microbiol.,* **8:** 99-145.

Babich, H. and Stotzky, G. (1983) Synergism between nickel and copper in their toxicity to microbes: Mediation by pH. *Ecotoxicol. Environ. Saftey.,* **7:** 576-587.

Bailliez, C., Largeau, C., Berkaloff, C. and Casadevall, C. (1986) Immobilization of *Botryococcus braunii* in alginate: Influence on chlorophyll content, photosynthetic activity and degradation during bach culture. *Appl. Microbial. Biotechnol.,* **23:** 316-366.

Ball, P.R., Shales, S.W. and Chopra, I. (1980) Plasmid-mediated tetracycline resistance in *Escherichia coli* involves increased efflux of antibiotics. *Biochem. Biophys. Res. Commun.* **93:** 74-81.

Banerjee, M., Misha, S. and Chatterjee, J. (2004) Scavenging of nickel and chromium toxicity in *Aulosira fertilissima* by immobilization: Effect on nitrogen assimilating enzymes. *Elecronic Res. J. of Micobial Biotech.,* **7:** (3) 55-76.

Barman, S.C., Sahu, R.K., Bhargava, S.K. and Chatterjee, C. (2000) Distribution of heavy metals in wheat, mustard and weeds grown in field irrigated with industrial pollutants. *Bulletin of environ. Contam. and Toxicol.,* **64:** 489-496.

Batterton, J.C. and Van Baalen, C. (1968) Phosphorus deficiency and phosphate uptake in the blue-green algae, *Anacystis nidulans. Can. J. Microbiol.,* **14:** 341-348.

Becker, E.W. (1986) Nutritional properties of micro algae: potentials and constraints. In: (ed. A. Richmond) LRC Handbook of micro-algal mass culture. *CRC Press, Inc. Boca Ranon, Florida, pp.* 339-376.

Beesley, C.E. (1994) The analysis of heterocyst specific sequences from the cyanobacterium. *Nostoc* PCC 6720. Ph.D. Thesis. Institute of environmental and Biological Sciences, University of Lancaster, U.K.

References

Bently-Mowart, J.A. and Reid, S.M. (1977) Survival of marine and phytoplankton in high concentrations of heavy metals and uptake of copper. *J. Exp. Mar. Biol. Ecol.,* **26:** 249-264.

Bergman, B., Rai, A.N. and Johanson, C. (1992) Cyanobacterial plant symbiosis. *Symbiosis,* **14:** 61-78.

Beverridge, T.J. and Doyle, R.J. (1989) Uptake of heavy metals in cyanobacteria. *John Wieley & Sons. New York.*

Bhaya, D., Schwaez, R. and Grossmann, A.R. (2000) Molecular response to environmental stress. In: *The ecology of Cyanobacteria* (eds. B.A. Whitton and M. Potts). *Kluwer Academic Publishers, Dordrecht, The Netherlands., pp.* 13-35.

Bienfait, H.F. and Van der Mark, F. (1983) Phytoferritin and its role in iron metabolism. *Ann. Proc. Phytochem. Soc.,* **21:** 111-123.

Bisen, P.S. (1984) Glucose metabolism in cyanobacterium *Nostoc* strain MAC-grown under photoautotrophic, photoheterotrophic and chemoheterotrophic regimes. *Acta Botanica Indica.,* **12:** 107-113.

Bisen, P.S. and Shanthy, S. (1991) Regulation of assimilatory *nitrate reductase* in the cyanobactewrium *Anabaena doliolum. Curr. Microbiol.,* **23:** 239-244.

Blackwell, K.J., Singleton, I. and Tobin, J.M. (1995) Metal cation uptake by yeast: A review. *Applied Microbiol.* and *Biotechnol.,* **43:** 579-584.

Blount, J.W., Dixon, R.A., Paiva, N. L. (1989) Stress response in alfalfa (*Medicago sativa*): Toxicity of Chromium and tin to *Anabaena doliolum*: Interaction with sulphur containing amino acids and thiols. *Biol. Met.*, **3** (1): 55-60.

Boggs, S.J., Livermore, D.G. and Seitz, M.G. (1985) Humic macromolecules in natural waters. *J. Macromol. Sci. Rev. Macromol. Chem. Phys.,* **25:** 599-567.

Bohme, H. (1998) Regulation of nitrogen fixation in heterocyst–forming cyanobacteria. Trends in *Plant Science.*, **3** (9): 346-351.

Bonaly, J., Bariand, A., Duret, S. and Mestre, J.C. (1980) Cadmium cytotoxicity and variation in nuclear content of DNA in *Euglena graciella. Plant physiol.*, **49:** 286-290.

Bone, D.H. (1971) *Nitrogenase* activity and nitrogen assimilation in *Anabaena flos-aquae* growing in continuous culture. *Arch. Microbiol.*, **80:** 234-241.

Bornet, E. and Flahault, C. (1886) A study of Nostocacean heterocysts. *Ann. Sci. Natl. Bot.*, **3:** 323-381.

Bothe, H. and Floener, L. (1980) Nitrogen fixation in *Rhopalodia gibba,* a diatom containing blue-greenish inclusions symbiotically. In: *Endosymbiosis and Cell Biology* (eds. W. Schwemmler and H.E.A. Schenk)., *pp.* 541-552.

Bothe, H. and Loos, E. (1972) Effect of far-red light and inhibitors on nitrogen-fixation and photosynthesis in blue-green algae *Anabaena cylindrica. Arch. Microbiol.*, **86:** 241-254.

Bothe, H. and Neuer, G. (1988) Electron donation to *nitrogenase* in heterocyst. In: *Methods in Enzymology.* Vol. 67. (eds. L. Packer and A.N. Glazer). *Academic Press Inc., San Diego., pp.* 496-501.

Bothe, H., Neuer, G., Kalbe, I. and Eisbrenner, G. (1980) Electron donors and *hydrogenase* in nitrogen-fixing microorganisms. In: *Nitrogen-fixation* (eds. W.D.P Stewart and J.R. Gallon). *Academic Press, London., pp.* 83-112.

Bottomley, P.J. and Stewart, W.D.P. (1976) ATP pools and transients in the blue-green algae *Anabaena cylindrica. Arch. Microbiol.*, **108:** 249-258.

Bottomley, P.J. and Van Baalen, C.C. (1978) Characteristics of heterotrophic growth in the blue-green algae, *Nostoc* strain *MAC. J.*, **11:** 54-76

Brachet, J. (1975) The effects of some inhibitors of RNA synthesis and proteolysis on morphogenesis in *Acetabularia mediterranea. Biochem. Physiol. Pflanzen.*, **168:** 493-510.

References

Bradeen, D.A., Winget, G.D., Gould, J.M. and Ort, D.R. (1973) Site-specific inhibition of photophosphorylation in isolated spinach chloroplasts by mercuric chloride. *Plant Physiol.,* **52:** 680-682.

Branue, W. (1980) Structural aspects of akinete germination in the cyanobacterium *Anabaena variabilis. Arch. Microbiol.,* **122:** 289-295.

Bristol, B.M. (1919) On the retention of vitality of algae from old stored soils. *New Phytol.,* **18:** 92-107.

Brodelius, P. (1984) Immobilized viable plant cells. *Ann. N.Y. Acad. Sci.,* **434:** 382-393.

Brodelius, P. (1985) The potential role of immobilization in plant cell biotechnology. *Trends of Biotechnol.,* **3:** 280-285.

Brodelius, P. and Mosbach, K. (1982) Immobilized plant cells. *Adv. App. Microbiol.,* **28:** 1-26.

Brouers, M. and Hall, D.O. (1985a) Hydrogen production, ammonia production and nitrogen fixation by free and immobilized cyanobacteria. In: *Energy from Biomass (*eds. *King's Coll., London)., pp.* 387-392.

Brouers, M. and Hall, D.O. (1985b) In energy from Biomass (eds. W. Paiz, J. Coombs and D.O. Hall). *pp.* 387-392.

Brouers, M. and Hall, D.O. (1986) Hydrogen and ammonia production by immobilized cyanobacteria. *J. Biotechnol.,* **3:** 307-321.

Brouers, M., Jong, H.D., Shi, D.J., and Hall, D.O. (1989) Immobilized cells: An appraisal of the methods and applications of cell immobilization techniques. In: *Algal and Cyanobacterial Biotechnology* (eds. R.C. Cresswell, T.A.V. Rees and N. Shah). *John Wiley and Sons, New York, pp.* 272-293.

Brown, C.N., Brown, M.D. and Meers, J.L. (1974) *In: Advances in Microbial Physiology* (eds. A.H. Ros and W. Tempest). *Academic Press, London, New York., pp.* 1-52.

Bryant, D.A. (1994) The molecular biology of cyanobacteria. *Kluwer Academic Publisher, Dordrecht, Netherlands.*

References

Bucke, C. (1983) Immobilized cells. *Phil. Trans. Roy. Soc. London, Ser. B.,* **30:** 360-389.

Campbell, P.M. and Smith, G.D. (1986) Transfer and accumulation of nickel ions in the cyanobacterium *Anabaena cylindrica. Arch. Biochem. Biophys.,* **244:** 470-477.

Cano, M., Zaccarode Mule, M.C., Caire, G. and Halperin, D. (1993) Bio-fertilizer of rice plants with the cyanobacterim *Tolypothix tenius. International Journal of Experimental Botany (Phytol).,* **53** (2): 149-155.

Canto de Loura, I., Dubacq, J.P. and Thomas, J.C. (1987) The effects of nitrogen deficiency on pigments and lipids of cyanobacteria. *Plant Physiol.,* **83:** 838-843.

Capone D.G., Zeh, J.P., Paerl, H.W., Bergman, B and Carpenter, E.J. (1997) *Trichodesmium,* a globally significant marine cyanobacterium. *Science.,* **276:** 1221-1229.

Carr, N.G. and Whitton, B.A. (1982) Cyanobacteria current perspectives. In: *The Biology of Cyanobacteria* (eds. N.G. Carr & B.A. Whitton). *Blackwell Scientific Publications, Oxford., pp.* 1-8.

Castenholz, R.W. and Waterbury, J.B. (1989) Oxygenic photosynthetic bacteria (Sect. 19) Group I. Cyanobacteria. In: *Begeys Manual of Systemic Bacteriology Vol. 3* (eds. J.T., Staley, M.P. Bryant, N. Pfenning and J.G. Holt). *Williams and Wilkins Co., Baltimore. pp.* 1710-1728.

Cedeno-Maldonedo, A., Swarder, J.A. and Health, R.L. (1972) The cupric ion as an inhibitor of photosynthetic electron transport in isolated chloroplasts. *Plant Physiol.,* **50:** 698- 701.

Chakrabarty, D., Mukharjee, S.C., Pati, S., Sengupta, M.K., Rahman, M.M. and Chowdhary, U.K. (2003) Arsenic groundwater contamination in middle Ganga plain, Bihar, India: A future danger. *Environ. Health Perspect.,* **111:** 1194-201.

Chakrabarty, D., Sangupta, M.K., Rahman, M.M., Ahemad, S., Chowdhary, U.K. and Hossain, M.A. (2004) Ground water arsenic contamination and its health effects on the Gnga-Bramhputra plain. *J. Environ. Monitor.* **6:** 74-83.

Chapman, J.S. and Meeks, J.C. (1983) Glutamine and glutamate transport by *Anabaena variabilis. J. Bacteriol.*, **156:** 122-129.

Chuan, M.C. and Liu, J.C. (1996) Release behavior of chromium from tannery sludge, *Water Research.*, **30:** 932-938.

Chau, Y.K. and Wong, P.T.S. (1975) Complexation of metals in natural waters. In: *Toxicity to Biota of Metal forms in Natural water* (eds. R.W. Andrew, P.V. Hodson and D.E. Konasewich). *International Joint Commission, Windsor, Ont., pp* 187-196.

Chau, Y.K., Gachter, R. and Lum-Shue Chan, K. (1974) Determination of the apparent complexion capacity of lake waters. *J. Fish Res. Bd. Can.*, **31:** 1515-1519.

Chevalier, P. and Noue, J.D.L. (1985) Wastewater nutrient removal with micro-algae immobilized in *Carrageenan. Enzyme Microb. Technol.*, **7:** 621-624.

Clark, R.L. and Jenson, T.E. (1969) Ultra-structure of akinete development in a blue-green algae *Cylindrospermum* sp. *Cytologia.*, **34:** 439-448.

Clarke, S.E., Stuart, J. and Sanders-Loeh J. (1987) Induction of siderophore activity in *Anabaena* sp. and its moderation of copper toxicity. *Appl. Environ. Microbiol.*, **53:** 917-922.

Clijsters, H. and Assche, F.V. (1985) Inhibition of photosynthesis by heavy metals. *Photosynth. Res.*, **7:** 31-40.

Cobb, H.D. and Myers, J. (1964) Comparative studies of nitrogen fixation and photosynthesis in *Anabaena cylindrica. Am. J. Bot.*, **51:** 753-766.

Cohen, D. and Parnas, H. (1976) An optimal policy for the metabolism of storage materials in unicellular algae. *J. Theor. Biol.*, **56:** 1-18.

Conway, H.L. (1978) Sorption of arsenic and cadmium and their effect on growth, micronutrient utilization and photosynthetic pigment composition of *Asterionella formosa. J. Fish. Res. Bd. Can.*, **35:** 286-294.

Conway, H.L. and Williams, S.W. (1979) Sorption of arsenic and cadmium and its effects on growth, micronutrient utilization and photosynthetic pigment composition of *Asterionella formosa. J. Fish. Res. Board, Landon.*, **36:** 579-586.

Cooke, D.T., Burden, R.S., Clarkson, D.T. and James, C.S. (1989) Xenobiotic-induced changes in membrane lipid composition: Effects on plasma membrane *ATPases.* In: *Mechanisms and Regulation of Transport Processes* (eds. R.K. Atkin and D.R. Clifford). *British Plant Growth Regulator Group.* Monograph No. 18, pp. 41-53.

Cossar, J.D., Rowell, S.R. and Stewart, W.D.P. (1984) Thioredoxin as modulators of *glucose-6-phosphate dehydrogenase* in a nitrogen fixing cyanobacterium. *J. Gen. Microbiol.*, **130:** 991-994.

Csaky, T.Z. (1948) On the estimation of bound hydroxylamine in biological materials. *Acta Chem Scand.*, **2:** 450-454.

Cseke, C., Balogh, A. and Farkas, G.H. (1981) Redox modulation of *glucose-6-phosphate dehydrogenase* in *Anacystis nidulans* and its uncoupling by phage infection. *FEBS Lett.*, **126:** 85-88.

Daniel, D. L., Kelly, D. and Budd, K. (2006) Biotransformation of mercury by cyanobacteria. *Journal of Phycol.*, **42:** 45-53.

Davies, A.G. (1978) Pollution studies with marine plankton. Heavy metals. *Adv. Mar. Biol.*, **150:** 381-508.

Davies, A.G. (1983) The effects of heavy metals upon natural marine phytoplankton populations. In: *Progress in Phycological Research* (eds. F.M. Round and D.J. Chapman). *Elsevier Science Pulishers, Amsterdam.*, **2:** 113-145.

Davies, A.G. (1988) Nutrients interactions in the marine environment. In: *Biochemistry of the Algae and Cyanobacteria.* (eds. L.J. Rogers and J.R. Gallon). *Clarendon Press, Oxford.* **28:** 241-256.

Day, J.C. and Codd, G.A. (1985) Photosynthesis and glucollate excretion by immobilized *Chlorella emersonii. Biotechnol. Lett.*, **7:** 573-576.

References

De Filippies, L.F. and Pallaghy, C.K. (1976) The effect of sub-lethal concentrations of mercury and zinc on *Chlorella. II.* Photosynthesis and pigment composition. *Z. Pflanzenphysiol.,* **78:** 314-322.

De Filippies, L.F. and Pallaghy, C.K. (1994) Heavy metal: Sources and biological effects. *pp.* 31-77.

De Filippies, L.F., Hampp, R. and Ziegler, H. (1981) The effect of sub-lethal concentrations of zinc, cadmium and mercury on *Euglena. II.* Respiration, photosynthesis and photochemical activities. *Arch. Microbiol.,* **128:** 407-411.

Delcourt, A. and Mestre, J.C. (1978) The effects of phenyl-mercuric acetate on the growth of *Chlamydomonas variablis* Dang. *Bull. Environ. Contamn. Toxicol.,* **20:** 145-148.

Desikachary, T.V. (1959) Cyanophyta. ICAR monograph on blue-green algae. New Delhi. *pp.* 686.

De Vasconcelos, L. and Fay, P. (1974) Nitrogen metabolism and ultra-structure in *Anabaena cylindrica.* The effect of nitrogen starvation. *Arch. Microbiol.,* **96:** 271-279.

Dodds, W., Gudder, D.A. and Mollenbauer, D. (1995) The ecology of *Nostoc* sp. *J. Phycol.,* **31:** 2-18.

Doolittle, W.F. (1979) The cyanobacterial genome, its expression and the control of that expression. *Adv. Microbiol. Physiol.,* **20:** 1-102.

Drbal, K., Veber, K. and Zahadnik, J. (1985) Toxicity and accumulation of copper and cadmium in the algae *Scenedesmus obliquus. Bull. Environ. Contam. Toxicol.,* **34:** 904-908.

Dubey, S.K. and Rai, L.C. (1987) Effect of chromium and tin on survival, *nitrogenase, nitrate reductase* and *glutamine synthetase* activities of *Anabaena doliolum. J. Plant Physiol.,* **130:** 165-172.

Dubois, M., Gilles, K.A., Hamiilton, J.K., Roberts P.A. and Smith, F. (1956) Chemical analysis of microbial cells. *In: Methods in Enzymology* (*eds.* J.R. Norris and D.W. Ribbons). *Academic Press, London.,* **5:** 272-274.

References

Dwivedi, C.P., Sinha, S.K., Ashaf, S.S., Verma, D.C. and Dubey, A.K. (2000) Application of Blue-green Algae biofertilizer in paddy cultivation under farm conditions in Uttar Pradesh. In: *Biofertilizers blue-green algae and Azolla* (eds. P.K. Singh, D.W. Dhar, S.L. Pabbi, R. Prasana and A. Arora), National Centre for Conservation and Utilization of Blue-green Algae, IARI, New Delhi, *pp.*1333.

Dwivedi, S., Misha, P.K., Rai, U.N., Tripathi, R.D., Susheela, M.R., Shina, S., Pal, A., Baghel, V.S. and Dwivedi, C.P. (2004) Fresh water cyanobacteria from thee agroclimatic zones of Uttar Pradesh, India: Distribution pattern with seasonal variation. *Journal of Environ. Biol.*, **26:** 1-10.

Ehlich, H.L. and Brierley, D.S. (1990) Microbial mineral recovery, *McGraw-Hill, New York.*

Eichorn, G.L. (1974) Active sites for biological macromolecules and their interaction with heavy metals. *In: Ecological Toxicology Research.* (ed. A.D. McIntyre and C.F. Mills). *Pelenum Press., New York.*

Epniewska, Z. and Bucior, K. (2001) Chromium contamination of soils, waters and plants in the vicinity of a tannery waste lagoon. *Environmental and Geochemical health.* **23:** 883-891.

Falkner, G., Horner, F. and Simonis, W. (1980). The regulation of energy dependent phosphate uptake by the blue-green algae, *Anacystis nidulans. Planta.,* **149:** 562- 569.

Fay, P. (1969) Metabolic activities of isolated spores of *Anabaena cylindrica. J. Exp. Bot.,* **20:** 100-109.

Fay, P. (1992) Oxygen relations of nitrogen-fixation. *Microbial Rev.,* **56:** 340-373.

Fay, P. and Cox, R.M. (1967) Oxygen inhibition of nitrogen fixation in cell-free preparation of blue-green algae. *Biochem. Biophys. Acta.,* **143:** 562-569.

Fay, P. and Van Baalen, C. (1987) The Cyanobacteria (eds. Fay, P. and Van Baalen, C.), *Elsevier Science Publishers, Amsterdam.*

Finden, D.A.S., Tippings, E., Jaworski, G.H.M and Reynols, Q.S. (1984) Light-induced reduction of natural iron (III) oxide and its relevance to phytoplankton. *Nature.*, **209:** 783-784.

Fisher, N.S. and Frood, D. (1980) Heavy metal and marine diatoms: Influence of dissolved organic compounds of toxicity and selection for metal tolerance among four species. *Mar. Bio.*, **59:** 85-93.

Fiske, C.H. and Subba Rao, Y. (1975) Colorimetric determination of phosphorus. *J. Biol. Chem.*, **66:** 374-400.

Flores, E. and Herrero, A. (1994) Assimilatory nitrogen metabolism and its regulation. In: *The molecular Biology of Cyanobacteria.* (ed. D.A. Bryant). *Kluwer Acedemic Publishers, Dordrecht, The Netherland., pp.* 487-517.

Fritsch, F.E. (1905) Structure of the investment and spore development in some Cyanophyceae. *Beih. Bot. Centralbl.*, **18:** 194-214.

Fogg, G.E. (1944) Growth and heterocyst production in *Anabaena cylindrica Lemm. New Phytol.*, **43:** 167-175.

Fogg, G.E. (1988) The flexibility and variety of algal metabolism. In: *Biochemistry of the algae and cyanobacteria* (eds. L.J. Rogers and J.R. Gallon). *Clarendon Press, Oxford.*, **28:** 3-12.

Fogg, G.E. and Than-Tun (1960) Interaction of photosynthesis and assimilation of elementary nitrogen in a blue-green algae. *Proc. R. Soc. B.*, **153:** 111-127.

Fogg, G.E., Stewart, W.P.D., Fay, P. and Walsby, A.E. (1973) The blue-green algae. *Academic Press. London., pp.* 35-37.

Foy, C.D., Chney, R.L. and White, M.C. (1978) The physiology of metal toxicity in plants. *Ann. Rev. Plant Physiol.*, **29:** 511-566.

Fukui, S. and Tanaka, A. (1982) Immobilized microbial cells. *Ann. Rev. Microbial.*, **36:** 145-172.

Fuhs, F.W. (1973) Cytochemical examination. In: *The Biology of Blue-green Algae.* (eds. N.G. Carr and B.A. Whitton). *University of California Press, Berkeley., pp.* 117-143.

Fujita, Y., Murakami, A., Aizawa, K. and Ohki, K. (1994) Short-term and long-term adaptation of the photosynthetic apparatus Homeostasis properties of thylakoids. In: *The Molecular Biology of Cyanobacteria.* (ed. D.A. Bryant). *Kluwer Academic Publishers, Dordrecht, The Netherlands., pp.* 677-692.

Gachter, R. (1976) Study of photosynthetic algae. *Z. Microbiol.,* **36:** 145-172.

Gadd, G.M. (1990) Heavy metal accumulation by bacteria and other microorganisms. *Experimentia.,* **46:** 834-840.

Gadd, G.M. and Griffith, A.J. (1978) Microorganisms and heavy metal toxicity. *Microbiol. Ecol.,* **4:** 303-317.

Gallon, J.R (1980) Nitrogen fixation by photoautotrophs. In: *Proceedings of an International Symposium on nitrogen fixation.* (eds. W.D.P. Stewart and J.R. Gallon). *Academic press, New York., pp.* 451.

Gallon, J.R., Chaplin, A.E., (1987) An introduction to nitrogen fixation. *Cassell Educational, London, U.K.*

Geddie, J.L., and Sutherland, I.W. (1993) Uptake of metals by bacterial polysaccharides. *J. Appl. Bacteriol.,* **74:** 476-472.

Geesey, G.G., and L.K. Jang (1989) Interactions between metal ions and capsular polymers. pp.325-357.

Georgiadis, M.M., Komiya, H., Chakrabarti, P., Wood, D., Kornue, J.J. and Rees, D.C. (1992) Crystallographic structure of the *nitrogenase* iron protein from *Azotobacter vinelandii. Science., (New York).* **257:** 1653-1659.

Gentile, J.H. and Maloney, T.E. (1969) Toxicity and environmental, requirements of a strain of *Aphanizomenon flos-aquae* (L.) *Ralfs. Can. J. Microbiol.,* **15:** 165-173.

Gileva, E.A. (1964) Dynamics of the accumulation of chemical elements by the algae *Cladophora fracta* and the dependence of the accumulation on the concentration of elements in solution. *Fiziol. Rast.,* **11:** 518-586.

Gipps, J.F. and Coller, B.A.W. (1980) Effect of physical and culture conditions on uptake of Cadmium by *Chlorella pyrenoidosa. Aus. J. Mar. Freshwater Res.*, **31:** 747-755.

Glazer, A.N. (1989) Light guides: directional energy transfer in a photosynthetic antenna. *J. Biol. chem.* **264:** 1-4.

Gleason, F.K. and Wood, J.M. (1988) Secondary metabolism in Cyanobacteria, In: *The cyanobacteria* (eds. P. Fay and C. Van Baalen). *Elsevier, Amsterdam, New York, Oxford., pp.* 437-449.

Golden, J.W., Robinson, S.J. and Haselkorn, R. (1985) Rearrangement of nitrogen-fixation genes during heterocyst differentiation in the Cyanobacterium, *Anabaena. Nature., (London).* **314:** 419-427.

Gomont, M. (1888) A study of filamentous cyanobacterium. *Bull. Soc. Bot., France.*, **35:** 204-236.

Gould, J.M. (1975) Inhibition of photosystem II-dependent photophosphorylation in chloroplast by mercury. *Res. Commun.*, **64:** 673-680.

Gourdon, R., Rus, E., Bhende, S. and Sofer, S.S. (1990) Mechanism of Cadmium uptake by activated sludge. *Appl. Microbiol., Biotechnol.*, **34:** 274-278.

Grillo, J.F. and Gibson, J. (1979) Regulation of phosphate accumulation in the unicellular cyanobacteria, *Synechococcus., J. Bacteriol.*, **140:** 508-517.

Guerero, M.G. and Lara C. (1987) Assimilation of inorganic nitrogen. In: *The cyanobacteria* (eds. P. Fay and C. Van Baalen). *Elsevier Science Publishers, London and New York., pp* 163-181.

Guerts van Kessel, A.H.M., Mullahy, J.H. and Lijkens, H.F. (1977) The relation of heterocyst and hormogonia to nitrogen fixation and reproduction in blue-green algae. *Plant Syst. Envol.*, **128:** 171-176.

Gupta, S.L. (1988) Copper inhibition of photosystem II activity in the cyanophage *AS-2-* resistant mutant. *J. Gen. Appl. Microbiol.*, **34:**1-5.

Gurudatta, G.U., Ghosh R., Nambir, K.M.R. and Singh, H.N. (1987) Characterization of *glutamate synthetase* and *ATPases* in a mutant strain of the cyanobacterium *Nostoc muscurum,* capable of metabolizing methylamine as a source of nitrogen. *J. Plant Physiol.,* **127:** 323-329.

Gutknecht, J. (1961) Mechanism of Radioactive zinc uptake by *Ulva lactuca. Limnol. Oceanogr.,* **4:** 426-431.

Habermann, H.M. (1969) Reversal of copper inhibition in chloroplast reactions by manganese. *Plant Physiol.,* **44:** 331-336.

Hader, D.P., Worrest, R.C. and Kumar, H.D. (1989) Aquatic ecosystems. UNEP environmental effects panel report., *pp.* 39-48.

Hall, D.O., Brouers, Shi, D.J. and Rao, K.K. (1985a) Photo-production of ammonia by immobilized cyanobacteria. In: *Proceedings of Workshop on Molecular Biosciences and Biotechnology.* Madras, *pp.*1-29.

Hall, D.O., Brouers, Shi, D.J. and Rao, K.K. (1985b) Photo-production of ammonia by immobilized cyanobacteria. In: *Abstracts. 5th International Symposium on Photosynthetic prokaryotes.* Grindelwald, *pp.* 263.

Hall, S.L. and Fisher, Jr. (1983) Organic compounds released from a natural population of epibenthic blue-green algae. *J. Physiol.,* **19:** 365-368.

Hampp, R., Beulich, K. and Ziegler, H. (1976) Effect of zinc and cadmium on photosynthetic CO_2 fixation and Hill activity of isolated spinach chloroplast. *Z. Pflanzen. Physiol.,* **77:** 336-344.

Hannan, P.J. and Patouillet, C. (1972) Effects of mercury on algal growth rates. *Biotechnol. Bioengineer.,* **14:** 93-101.

Harding, J.P.C. and Whitton, B.A. (1976) Resistance to zinc of *Stigeoclonium tenue* in the field and the laboratory. *Br. Phycol. J.,* **11:** 417-427.

Hargreaves, J.W. and Whitton, B.A. (1976 a) Effects of pH on growth of acid stream algae. *Br. Phycol. J.,* **11:** 215-223.

Hargreaves, J.W. and Whitton, B.A. (1976 b) Effects of pH on tolerance of *Hormidium rivulare* to zinc and copper. *Oecologia.*, **26:** 235-243.

Harold, F.M. (1966) Inorganic polyphosphates in biology structure metabolism and function. *Bacteriol. Rev.*, **30:** 772-794.

Harris, R., White, D.B. and Macfarlane, R.B. (1970) Mercury components reduce photosynthesis by plankton. *Science,* **170:** 736-737.

Harsted-Romeo, M. and Gnassia-Barelli, M. (1980) Effect of complexation by natural phytoplankton exudates on the accumulation of cadmium and copper by Haptophyceae. *Marine Biol.*, **59:** 79-84.

Haselkorn, R. (1978) Heterocyst. *Ann. Rev. Plant Physiol.*, **29:** 319-344.

Haselkorn, R. (1991) Genetic systems in cyanobacteria. In: *Methods in Enzymology.* **204:** 418-430.

Havlik, B., Stasy, J., Prasilova, J., Kratzer, K. and Hanusova, J. (1979) Mercury circulation in aquatic environment. Part-2. Metabolism of methyl and phenyl-mercury in phytoplankton. *Acta Hydrochem. Hydrobiol.*, **7:** 401-408.

Haystead, A., Robinson, R. and Stewart, W.D.P. (1970) *Nitrogenase* activity in extracts of heterocystous and non-heterocystous blue-green algae. *Arch. Microbiol.*, **74:** 235-245.

Healey, F.P. (1973) Characteristics of phosphorus deficiency in *Anabaena nidulans. J. phycol.*, **9:** 383-394.

Healey, F.P. (1982) Phosphate. In: The Biology of Cyanobacteria (eds. N.G. Carr and B.A. Whitton). *Blackwell Sci. Publ., Oxford , pp.* 105-124.

Henriksson, L.E. and Da Silva, E.J. (1978) Effects of some inorganic elements on nitrogen fixation in blue-green algae and some ecological aspects of pollution. *Z. Allge. Microbiol.*, **18:** 487-494.

Herbert, D.P., Phipps, P.J. and Strange, R.E. (1971) Chemical analysis of microbial cells. In: *Methods in microbiology, 5th B.* (eds.

J.R. Norris and D.W. Ribbons). *Academic Press, New York and London., pp.*209-344.

Hewitt, E.J. and Nicholas, D.J. (1963) Cations and ions: inhibitions and interactions in metabolism and in enzyme activity. In: *Metabolic inhibitors.* (eds. R.M. Hochter and J.H. Quastel). *Academic Press, New York and London, pp.* 311-436.

Hirosawa, T. and Wolk, C.P. (1979a) Factors controlling the formation of akinetes adjacent to heterocysts in the cyanobacterium *Cylindrospermum licheniforme* Kutz. *J. Gen. Microbiol.,* **114:** 423-432.

Hirosawa, T. and Wolk, C.P. (1979b) Isolation and characterization of a substance which stimulates the formation of akinetes in the cyanobacterium *cylindrospermum licheniforme* Kutz. *J. Gen. Microbiol.,* **114:** 433-441.

Hodson, P.V., Borgmann, U. and Shear, H. (1979) Toxicity of copper to aquatic biota. In: *Copper in the Environment.* Part II. Health Effects (ed. J.O. Nriagu). *John Wieley and Sons, New York., pp.* 307-372.

Horichu, T., Horichu, S. and Mizuno, D. (1959) A possible negative feedback phenomenon controlling formation of *alkaline phosphomonoesterase* in *Escherichia coli. Nature (Lond.),* **183:** 1529-1530.

Horne, A.J. and Goldman C.R. (1974) Suppression of nitrogen fixation by blue-green algae in eutrophic lake with trace additions of copper. *Science (New York),* **183:** 409-411.

Huntsman, S.A. and Sunda, W.G. (1980) The role of trace metals in regulating phytoplankton growth with emphasis on Fe, Mn and Cu. In: The physiological Ecology of Phytoplankton, Studies in Ecology (ed.1st Morris). *Blackwell Sci. Publ., Oxford.,* **7:** 283-328.

Husaini, Y., Singh, A.K. and Rai, L.C. (1991) Cadmium toxicity to photosynthesis and associated electron transport system of *Nostoc linckia. Bull. Environ. Contam. Toxicol.,* **46:** 146-150.

Hustichinson, T.C. (1973) Comparative studies of the phytotoxicity of heavy metals to phytoplankton and their synergestic interactions. *Water Pollut. Res. Can.,* **8:** 68-69.

Ihlenfeldt, M.J.A. and Gibson, J. (1975) Phosphate utilization and *alkaline phosphatase* activity in *Anacystis nidulans* (*Synechococcus*). *Arch. Microbiol.*, **102:** 23-28.

Ikemoto, H. and Mitsui, A. (1983 a) Continuous indoor and outdoor hydrogen photo-production from sulphide by immobilized marine photosynthetic sulphur bacterium *Chomatium* sp. Miami PBS 1071. In: *Proc. 6th International Congress on Photosynthesis. Brussels, Belgium,* **1:** 413.

Ikemoto, H. and Mitsui, A. (1983 b) Continuous hydrogen photo-production from sulphide by an immobilized marine photosynthetic bacterium *Chomatium* sp. Miami PBS 1071. In: *Advances in Photosynthetic Research,* Vol. 2nd (ed. C. Sybesma). *Martinus Nijhoff / Dr. W. Junk Publishers, The Hague, Boston-Lancaster., pp.* 789-792.

Inthorn, D., Incharoenzaka, A. and Sidtitoun, N. (2001) Removal of mercury, cadmium and lead in aqueous solution by micro-algae. *Asian Journal of Microbiology, biotechnology and Environmental Sciences (AJMBES).*, **3:** 109-116.

Ip, S.M., Rowell, P. and Stewart, W.D.P. (1983) The role of specific cations in regulation of cyanobacterial *glutamine synthetase. Biochem. Biophys. Res. Commun.*, **114:** 206-213.

Irmer, U., Wachholz, I., Schafer, H. and Lorch, D.W. (1986) Influence of lead on *Chlamydomonas reinhardii.* (volvocales, chlorophyta): Accumulation, toxicity and ultra-structure changes. *Environ. Exp. Bot.*, **26:** 97-105.

Itoh, M., Yuasa, M. and Kobayashi, T. (1975) Adsorption of metal ions on yeast cells at varied concentration. *Plant Cell Physiol.*, **16:** 1167-1169.

Jackson, G.A. and Morgan, J.J. (1987) Trace metal-chelator interactions and phytoplankton growth in Sea water media. Theoretical analysis and comparison with reported observations. *Limnol. Oceanogr.*, **13:** 268-282.

James, B.R. (1996) The Challenging of remediating chromium contaminated soil. *Environ. Sci. and Technol.*, **29:** 1-44.

References

Janezewski, E. (1874) A study of cyanobacteria. *Ann. Sci. Natl. Bot. Ser.*, **519:** 119-130.

Jensen, T.E. (1984) Cell inclusions in the cyanobacteria. *Ann. Rev. NewYork., Acad. Sci.*, 279-282.

Jensen, T.E. (1985a) Cyanobacterial inclusions of irregular occurrence: systematic and evolutionary implications. *Cytobios.*, **39:** 35-62.

Jensen, T.E. (1985b) Cell inclusions in the cyanobacteria. *Arch. Hydrobiol. Suppl.*, **71:** 33-73.

Jensen, T.E. and Sicko, L.M. (1974) Phosphate metabolism in blue-green algae. I. Fine structure of the polyphosphate over-plus phenomenon in *Plectonema boryanum. Can. J. Microbiol.*, **20:** 1235-1239.

Jensen, T.E., Baxter, M., Rachlin, J.W. and Jani, V. (1982) Uptake of heavy metals by *Plactonema boryanum* (Cyanophyceae) into cellular component, especially polyphosphate bodies: An X-ray energy dispersive study. *Environ. Pollut.*, **27:** 119-127.

Jensen, T.E., Baxter, M., Rachlin, J.W. (1982) Uptake of heavy metals by *Plactonema boryanum* (Cyanophyaceae) into cellular components especially polyphosphate bodies on X-ray energy dispersive study. *Environ; Pollut. Ser. A.*, **27:** 119-127.

Johanson, P.E. and Shubert, L.E. (1986) Accumulation of mercury and other elements by *Spirulina* (Cyanophyceae). *Nutr. Rep. Int.*, **34:** 1063-1070.

Kallas, T., Rippka, R., Coursin, T., Rebiere, M.C., Tandeau de Marsac, N. and Cohen-Bazire, G. (1994) Aerobic nitrogen fixation by non-heterocystous cyanobacteria. In: *Photosynthetic prokaryotes; cells differentiation and function.* (eds. G.C. Papageogious and L. Packer). *Elsevier, New York., pp.* 281-303.

Kallqvist, T. and Meadows (1978) The toxic effects of copper on algae and rotifers from a soda lake (Lake Nakuru, East Africa). *Water Res.*, **12:** 771-777.

Kamp-Nielesen, L. (1971) The effect of deleterious concentrations of mercury on photosynthesis and growth of *Chlorella pyrenoidosa. Plant physiol.*, **24:** 556-561.

Karpunina, L.V., Nikitina, V.E., Vorotilova, N.A., Konovalova, N.G. and Shataeva, L.K. (1989) The study of nitrogen fixing activity of *Azospirillum brasilense* sp. 7 cells immobilized on macro-porous absorbents. *Biotechnol.*, **5:** 208-211.

Katoh, S. and Takamiya, A. (1964) Nature of copper protein binding in spinach plastocyanin. *J. Biochem.*, **55**: 556-561.

Katoh, S. Shiratori, and Takamura, T. (1962) Purification and some properties of spinach plastocyanin. *J. Biochem.*, **51:** 32-40.

Kelly, M.G. and Whitton, B.A. (1989 a) Relation between accumulation and toxicity of zinc in *Sigeoclonium* sp. (Chaetophorales, Chlorophyta). *Phycologia.*, **28 :** 512-517.

Kelly, M.G. and Whitton, B.A. (1989 b) Inter-specific differences in Zn, Cd and Pb accumulation by freshwater algae and bryophytes. *Hydrobiologia.*, **175 :** 1-11.

Kerby, N.W., Musgrave, S.C., Rowell, P., Shestakov, S.V. and Stewart, W.D.P. (1986) Photoproduction of ammonia by immobilized mutant strains of *Anabaena variabilis. Appl. Microbiol. Biotechnol.*, **24:** 42-46.

Kerndorff, H. and Schnitzer, M. (1980) Sorption of metals on humic acid. *Geochem. Cosmochem. Acta.*, **44:** 1701-1708.

Kessler, E. (1986) Limits of growth of five *Chlorella* species in the presence of toxic heavy metals. *Arch. Hydrobiol.*, **73:** 123-128.

Khoja, T.M. and Whitton, B.A. (1971) Heterotrophic growth of blue green algae. *Arch. Microbiol.*, **79:** 280-282.

Khummongkol, D., Canterford, G.S. and Fryer, C. (1982) Accumulation of heavy metals in unicellular algae. *Biotechnol. Bioeng.*, **24:** 2643-2660.

Kimbrough, D.E., Cohen, Y., Winer, A.M.. Creelman, L., and Mabuni, C. (1999) *A critical Review of Environ. Sc. and Technol.,* **29:** 1-44.

Kingdon, H.S., Hubbard, J. and Sutman, E.R. (1968) Biochemistry., **7:** 2136-2142.

Kratz, W.A. and Myers, J. (1955) Nutrition and growth of several blue-green algae. *Amer. J. Bot.,* **42:** 282-287.

Kumar, A., Sinha, R.P. and Hader, D.P. (1996) Effect of UV-B on enzyme of nitrogen metabolism in the cyanobacterium *Nostoc calcicola. J. Plant Physiol.,* **148:** 86-91.

Kurek, E., Czaban, J. and Bollag, J. (1982) Sorption of Cd by microorganisms in competition with other soil constituents. *Appl. Environ. Microbiol.,* **43:** 1011-1015.

Kuwabara, J.S. (1985) Phosphorus-zinc interactive effects on growth by *Selenastrum capricornutum* (Chlorophyta). *Environ. Sci. Technol.,* **19:** 417-421.

Laegried, M., Alstad, J., Klaveneness D. and Selp, H.M. (1983) Seasonal variation of cadmium toxicity towards the algae *Selenastrum capricornutum* printz in two lakes with different humus content. *Environ. Sci. Technol.,* **17:** 357-361.

Lambert, G.R., Daday, A. and Smith, G.D. (1979) Hydrogen evolution from immobilized cultures of the cyanobacterium *Anabaena cylindrica B 629. FEBS Lett.,* **101:** 125-128.

Lang, N.J. (1968) The fine structure of blue-green algae. *Ann. Rev. Microbiol.,* **22:** 15-46.

Lange, W. (1971) Limiting nutrients elements in filtered Lake Eric Water Res., **5:** 1031-1048.

Lange, W. (1974) Chelating agents and blue-green algae. *Can. J. Microbiol.,* **20:** 1311-1321.

Larsson, C.M. and Olsson, T. (1979) Firefly assay of adenine nucleotide from algae: Comparison of extraction methods. *Plant Cell Physiol.,* **20:** 145-155.

Laube, V. M., McKenzie, C.N. and Kushner, D.J. (1980) Strategies of response to copper, cadmium and lead by a blue green and a green algae. *Can. J. Micribiol.,* **26:** 1300-1311.

Lawry, N.H. and Simon, R.D. (1982) The normal and induced occurrence of cyanophycean inclusion bodies in several blue-green algae. *J. Phycol.,* **18:** 391-399.

Lee, L.H. and Lustigman, S.R. (2002) Combined effect of mercuric chloride and selenium dioxide on the growth of the cyanobacteria *Anacystis nidulans. Bull. Environ. Contam. Toxicol.,* **69:** 900-901.

Lee, L.H., Lustigman, B., Muray, S. and Koepp, S. (1999) Effect of selenium on the growth of cyanobacterium *Anacystis nidulans. Bull. Environ. Contam. Toxicol.,* **49:** 272-278.

Lefebvre, D.D., Kelly, D. and Kenneth, B. (2006) Biotransformation of Hg (II) by Cyanobacteria. *J. Phycol.,* **42:** 45-53.

Leganges, F., Fernandez-Pinas, F. and Wolk. C.P. (1994) Two mutations that block heterocyst differentiation have different effects on akinete differentiation in *Nostoc ellipsospermum. Mol. Microbiol.,* **12:** 679-684.

Lehman, M. and Wober, G. (1976) Accumulation, mobilization and turnover of glycogen in the blue-green algae *Anacystis nidulans. Arch. Microbiol.,* **11:** 93-97.

Les, A. and Walker, R.W. (1984) Toxicity and binding of copper, zinc and cadmium by the blue-green algae, *Choococcus paris, Water, Air, Soil Pollut.,* **23:** 129-139.

Lester, J.N., Steriff, R.M., Rudd, T. and M.J. Brown (1984) Assessment of algal and bacterial extra-cellular polymers in controlling metal removal in biological wastewater treatment., *pp.*197-217.

Levine, R.P. (1969) The analysis of photosynthesis using mutant strains of algae and higher plants. *Ann. Rev. Plant Physiol.,* **20:** 523-540.

Levinson, H.S. and Maahler, I. (1998) *Phosphatase* activity and lead resistance in *Citrobacter freundii* and *Naphylococcus aurens. FEMS. Microbiol Litt.,* **161:** 135-138.

Levinson, H.S., Maahler, I., Blackwelder, P. and Hood, T. (1996) Lead resistance and sensitivity in *Staphylococcus qureus. FEMS Microbial Technol.,* **145:** 421-425.

Li, M.K.W. (1979) Cellular composition and physiological characteristics of the diatom, *Thalassiosira weissflogii* adapted to cadmium stress. *Mar. Biol.,* **55:** 171-180.

Lindblad, P., Atkins, C.A. and John, S.P. (1991) Nitrogen-fixation by freshly isolated *Nostoc* sp from coralloid roots of the Cycad *Macrozamia reedlei (Fisch. ex. Guud) Gardn. Plant Physiol.,* **95:** 753-759.

Lipman, C.B. (1941) The successful revival of *Nostoc commune* from a herbarium specimen eighty seven years old. *Bull. Torrey Bot. Club.,* **68:** 664-666.

Livingstone, D. and Jaworski, G.H.M. (1980) The viability of akinetes of blue-green algae recovered from the sediments of Rostherne Mere. *Phycol. J.,* **15:** 357-364.

Livingstone, D. and Whitton, B.A. (1984) Water chemistry and *phosphatase* activity of the blue green alga *Rivularia* in upper Teesdale streams. *J. Ecol.,* **72:** 405-421.

Livingstone, D. Khoja, T.M. and Whitton, B.A. (1983) Influence of phosphorus on physiology of a hair forming blue-green algae (*Calothix parietina*) from an upland stream. *Phycologia.,* **22:** 345-350.

Livingstone, D. Khoja, T.M. and Whitton, B.A. (1984) Water chemistry and phosphate activity of blue-green algae *Rivularia* in upper Teesdale streams. *J. Ecol.,* **72:** 405-421.

Lockau, W. and Pfeffer, S. (1982) A cyanobacterial *ATPase* distinct from the coupling factor of photophosphorylation. *Z. Naturoforsch.,* **37c:** 658-664.

Lohmeyer, M. and Frierich, C.G. (1987) Nickel transport in *Alcalgenes eutrophus. Arch. Microbiol.,* **149:** 130-135.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with Folin-phenol reagent. *J. Biol. Chem.,* **193:** 265-276.

Lustigman, B., Lee, L.H., Morata, J. and Khan, F. (2000) Effects of thallium on the growth of *Anacystis nidulans* and *Chlamydomonas reinhardtii. Bull. Environ. Contam. Toxicol.*, **64:** 565-573.

Macaskie, L.E. and Dean, A.C.R. (1990) Metal sequestering biochemical in biosorption of heavy metals. B. Valesky, pp.199-248, *CRC Press, Boca Raton., Florida.*

Mackinney, G. (1941) Absorption of light by chlorophyll solutions. *J. Biol. Chem.* **140:** 315-322.

Mac Lean, E.I., Lucis, O.H. Shaikh, Z.A. and Zonsz, E.R. (1972) The uptake and subcellular distribution of Cd and Zn in microorganisms. Fed. Proc., **31:** 699-702.

Mak, A.H. and Trevan, M.D.C. (1988) Urea as a nitrogen source for immobilization *Chlorella.* In: *Biochemistry of the algae and cyanobacteria.* (eds. L.J. Rogers and J.R. Gallon). *Clarendon Press, Oxford.* **28:** 362-379.

Mallick, N., Pandey, S., Rai, L.C. (1994) Involvement of a low molecular weight protein in regulation of cadmium toxicity in the diazotrophic cyanobacterium *Anabaena doliolum. Biometals.*, **7:** 299-304.

Matsunaga, T. and Mitsui, A. (1982) Sea water based hydrogen production by immobilized marine photosynthetic bacteria. In: *Proc. Symp. Biotech. Bioeng.*, **12:** 441-450.

Matthijsh, C.P. and Lubberding, H.J. (1988) Dark respiration in cyanobacteria In: *Biochemistry of the algae and cyanobacteria.* (eds. L.J. Rogers and J.R. Gallon). *Clarendon Press, Oxford.*, **28:** 131-145.

McEldowney, S., Hardman, D.J. and Stephan, W. (1993) Pollution, Ecology and Bio-treatment. *Longman Scientific and Technical Publishing., New York.*

McHardy, B.M. and George, J.J. (1990) Bioaccumulation and toxicity of zinc in the green algae, *Cladophora glomerata. Environ.Pollut.*, **66:** 55-66.

McKnight, D.M. and Morel, F.M.M. (1979) Release of week and strong copper-complexing agent by algae. *Limnol. Oceangr.*, **24:** 823-837.

Mekinney, G. (1941). Absorption of light by Chlorophyll solutions, J. *Biol. Chem.*, **140**: 315-322.

Merrick, M.J. and Edwards, R.A. (1995) Nitrogen control in bacteria. *Microbial Rev.*, **59:** 604-622.

Mierle, G. (1982) Studies on the uptake and toxicity of copper and other transition metal ions with green algae, *Scenedesmus acuminates. J. Gen. Physiol.*, **69:** 71-75.

Mierle, G. and Stokes, P.M. (1976) Heavy metal tolerance and metal accumulation by planktonic algae. In: *Trace Substances in Environmental Health* (ed. D.D. Hemphill). Univ. Missouri. Columbia., **11:** 113-122.

Miller, M.M. and Lang, N.J. (1968) The fine structure of akinete formation and germination in *Cylindrospermum. Arch. Microbiol.*, **60:** 303-313.

Mitsui, A., Matsunaga, T., Ikemoto, H. and Renuka, B.R. (1985) Organic and inorganic waste treatment and simultaneous photoproduction of hydrogen by immobilized photosynthetic bacteria. *Dev. Indust. Microbiol.*, **26:** 209-222.

Mohamed, A.Z. (2001) Removal of Cd and Mn by a non-toxic strain of fresh water cyanobacterium *Gloeheco magna. J. Water Res.*, **35:** 4405-4409.

Mohanty, N., and Mohanty, N. (1988) Cation effects on primary processes of photosynthesis. In: *Advances in Frontier Areas of Plant Biochemistry* (eds. R. Singh and S.K. Sawhney). *Prentice-Hall, New Delhi., pp.* 1-18.

Mohanty, N., Vass, I. and Demeter, S. (1989) Copper toxicity affects photosystem-II electron transfer at the secondary quinone accepter. *Plant Physiol.*, **90:** 175-179.

References

Mora, B. and Fabregas, J. (1980) The effect of inorganic and organic mercury on growth kinetics of *Nitzschia acicularis* and *Tetraselmia suecia. B. Biol.,* **6:** 373-380.

Muro-Pastor A.M., Valladares, A., Flores, E. and Herrero, A. (1999) The *het C* gene is a direct target of *Ntc* A transcriptional regulator in cyanobacterial heterocyst development. *J. Bacteriol.,* **181:** 6664-6669.

Murray, M. and Horne, A.J. (1980) Regulation of *nitrogenase* activity in blue-green algae. Salinity Engineering Research Laboratory, *Rep. 80-5, Univ. California, Berkeley CA., pp.* 200-225.

Murthy, S.D.S., Bukhov, N.G. and Mohanthy, P. (1990) Mercury induced alternation of chlorophyll *a* fluorescence Kinetics in Cyanobacteria: Multiple effect of mercury on electron transport. *J. Phytochem. Phytobiol. B. Biol.,* **6:** 373-380.

Murthy, S.D.S., Bukhov, N.G. and Mohanthy, P. (1991) Mercury induces alteration of energy transfer in phycobilisomes by selectively affecting the pigment protein, phycocyanin in the cyanobacterium, *Spirullina platensis. Plant Cell Physiol.,* **32:** 213-217.

Murthy, S.D.S., Sabat, S.C. and Mohanthy, P. (1989) Mercury induced inhibition of photosystem-II activity and changes in the emission of fluorescence from phycobilisomes in intact cells of the cyanobacterium, *Spirulina platensis. Plant Cell Physiol.,* **30:** 1153-1157.

Musgrave, S.C., Kerby, N.W., Codd, G.A. and Stewart, W.D.P. (1982) Sustained ammonia production in immobilized filaments of the nitrogen-fixing cyanobacteria *Anabaena* 27893. *Biotechnol. Lett.,* **4:** 647-652.

Myers, J. and Kratz, W.A. (1955) Relationship between pigment content and photosynthetic characteristic in blue-green algae. *J. Gen. Physiol.,* **39:** 11-12.

Nalewajako, C. and Paul, B. (1985) Effects of manipulations of aluminum concentrations and pH on the phosphate uptake and photosynthesis of planktonic communities of two Precambrian shield lakes. *Can. J. Fish. Aquat. Sci.,* **42:** 1946-1953.

Narro, M.L. (1985) Oxidation of aromatic hydrocarbons by marine cyanobacteria. *The University of Texas Austin, Texas.*

Nichols, J.M. and Adams, N.G. (2000) Akinetes. In: *The Biology of Cyanobacteria* (eds. N.G. Carr and B.A. Whitton). *Blackwell Scientific, Oxford., pp.* 387-412.

Noda, H. and Horiguchi, S.P. (1971) Biochemical studies on marine algae. *Bull. Lap. Soc. Fish.*, **37** (1): 992-995.

Norris, P.R. and Kelly, D.P. (1977) Accumulation of Cadmium and cobalt by *Sacchomyces cerevisiae. J. Gen. Microbiol.*, **99:** 317-322.

Nriagu, J.O., Lawson, G., Wang, H.K.T. and Cheam, V. (1976) Dissolved trace metals in lakes superior, Erie and Ontario., *Environ. Sci. Technol.*, **30:** 178-187.

Ohnishi, T., Gall, R.S. and Mayer, M.L. (1975) An improved assay of inorganic phosphate in the presence of extra-labile compounds. Application to the *ATPase* assay in the presence of phosphocreation. *Anal. Biochem.*, **69:** 261-267.

Olfson, R.W., Loya, S. and Sim, R.G. (1979) Prokaryotic *metallothionein*: Preliminary characterization of a blue-green algal heavy metal-binding protein. *Biochem. Biophys. Res. Commun.*, **90:** 1025-1031.

Olfson, R.W., Loya, S. and Sim, R.G. (1980) Pysiological parameters of Prokaryotic *metallothionein* induction. *Biochem. Biophys. Res. Commun.* **95:** 1495-1503.

Orr, J. and Haselkorn, R. (1982) Regulation of *glutamine synthetase* activity and synthesis in free-living and symbiotic *Anabaena sp. J. Bacteriol.*, **152:** 626-635.

Overnell, J. (1975) The effect of heavy metals on photosynthesis and loss of cell potassium in two species of marine algae. *Marine Biol.*, **29:** 99-103.

Owers-Narhi, L., Robinson, S.J., De Rao, C.S. and Yacum, C.F. (1979) Reconstitution of Cyanobacterial photophosphorylation by a latent Cd^{+2} *ATPase. Biochem. Biophys., Res. Commun.*, **90:** 1025-1031.

References

Pandey, P.K. (1992) Investigation of heavy metal uptake in the cyanobacterium *Nostoc calcicola* Breb. Ph.D. Thesis. Centre of advanced study in Botany, Banaras Hindu University, Varanasi, India.

Pandey, P.K., and Singh, S.P. (1993) Hg^{+2}-uptake in a cyanobacterium. *Curr. Microbiol.,* **26:** 155-159.

Pandey, P.K., Saxena, R.K., and Bisen, P.S. (2002) Immobilization results in sustained calcium transport in *Nostoc calcicola. Curr. Microbiol.,* **44:** 173-177.

Pandey, P.K., Singh, B.B., Mishra, R.R. and Bisen, P.S. (1996) Ca^{2+}-uptake and its regulation in the cyanobacterium. *Curr. Microbiol.,* **26:** 155-159.

Pandey, P.K., Singh, C.B. and Singh, S.P. (1992) Cu uptake in a cyanobacterium: Fate of selected photochemical reactions. *Curr. Microbiol.,* **24:** 35-39.

Papageorgiou, G.C. (1987) Immobilized photosynthetic microorganisms. *Photosynthetica.,* **21:** 367-383.

Papageorgiou, G.C. and Lagoyanni, T. (1986) Immobilization of photosynthetically active cyanobacteria in glutaraldehyde-cross linked albumin matrix. *Appl. Microbiol. Biotechnol.,* **23:** 417-427.

Papageorgiou, G.C., Kalosaka, K., Sotiropoulou, G., Barbotin, J.N., Thomasset, B. and Thomas, D. (1988) Entrapment of active ion-permeable cyanobacteria (*Anacystis nidulans*) in calcium alginate. *Appl. Microbiol. Biotechnol.,* **29:** 565-571.

Passow, H. and Rothtein, A. (1960) The binding of mercury by the yeast cell in relation to changes in permeability. *J. Gen. Phyiol.,* **43:** 621- 633.

Passow, H., Rothtein A. and Clarkson, T.W. (1961) The general pharmacology of heavy metals. *Pharmacol. Rev.,* **13:** 185-224.
Patterson, M.L.G. (1996) Biotechnological applications of cyanobacteria. *J. Sci. Ind. Res.,* **55:** 669-684.

Payne, S.M. (1988) Iron and virulence in the family *enterobacteriacae CRC.*

Peschek, G.A. (1982) Nitrate and *nitrite reductase* and *hydrogenase* activity in *Anacystis nidulans* grown in Fe-and Mo-deficient media. *FEMS. Microbiol. Lett.*, **6:** 371-374.

Peschek, G.A., Schmetterer, G. and Wagesreiter, H. (1979) Oxidation of exogenous C-type cytochromes by intact spheroplasts of *Anacystis nidulans. Arch. Microbiol.*, **133:** 222-224.

Peter, J.N. and Sherman, L.A. (1978) Isolation and characterization of photosystem I and II membrane particles from the blue-green algae, *Synechococcus cedrorum. Biochem. Biophys. Acta,* **503:** 343-361.

Peterson, T., Falk, K.E., Leong, S.A., Klein, M.P. and Neilands, J.B. (1996) structure and behavior of spemidine siderophores. *J. Am. Chem. Soc.*, **102:** 7715-7718.

Petroy, R.A. and Bassham, J.A. (1973) Efficiency of energy conservation by aerobic glucose metabolism in *Aphanocapsa* 6714. *J. Bacteriol.*, **115:** 937-942.

Pettersson, A. and Bergman, B. (1989) Effects of aluminiumon ATP pools and utilization in the cyanobacterium *Anabaena cylindrica:* A model for the *in vivo* toxicity. *Plant Physiol.*, **76:** 527-534.

Pettersson, A., Hallbom, L. and Bergman, B. (1985) Physiological and structural response of the cyanobacterium *Anabaena cylindrica* to aluminum. *Plant Physiol.*, **63:** 153-177.

Pettersson, A., Hallbom, L. and Bergman, B. (1986) Aluminum uptake by *Anabeana cylindrica. J. Gen. Microbiol.*, **132:** 1771-1774.

Pettersson, A., Hallbom L. and Bergman, B. (1988) Aluminum effects on uptake and metabolism of phosphate in *Anabaena cylindrica. Plant Physiol.*, **86:** 112-116.

Pettersson, A., Hallbom, L., Kunst, L., Bergman, B., Roomans, G.M. and Bergman, B. (1985) Accumulation of aluminum by *Anabaena cylindrica* into polyphosphate granules: an X-ray energy-dispersive micro-analysis study. *J. Gen. Microbiol.*, **131:** 2545-2548.

Pickett, A.W., Carter, I.S. and Dean, A.C.R. (1975) Enzymatic activities of Cadmium and zinc-tolerant strain of *Klebsiella* sp. growing in glucose-limited chemostat. *Microbios.*, **15:** 105-111.

References

Plesincar, M. and Bendall, D.S. (1970) The plastocyanin content of chloroplasts from some higher plants estimated by a sensitive enzymatic assay. *Biochem. Biophys. Acta.*, **216:** 192-199.

Plowman, J.E., Loehr. T.M., Goldman, S.J. and Sanders-Loehr, J. (1984) Structure and siderophore activity of ferric schizokinen. *J. Inorg. Biochem.*, **20:** 183-197.

Polivka, T., Zigmantas, D., Frank, H.A., Bautista, J.A., Herek., Y., Koyama, J.L., Fujii, R., Sundstrom, V. (2001) Near-Infrared time-resolved study of the S-1 state dynamics of the caretenoid spheroidene. *J. Phys. Chem. B.*, **105** (5): 1072-1080.

Polivka, T., Zigmantas, D., Herek, S.K., Zigmantas, H.E., Akerlund, V. and Sundstrom, H. (1999) Direct observation of the (forbidden) S-1 state in caretenoids. *Proc. Natl. Acad. Sci., USA.*, **96** (9): 4914-4917.

Postgate, J.R. (1999) The fundamentals of nitrogen-fixation. *Cambridge University Press, London.*, **7:** 225-252.

Potts, M. and Morrison, S. (1986) Shifts in the intracellular ATP pool of immobilized *Nostoc calcicola* cells (cyanobacteria) induced by water stress. *Plant and Soil.*, **90:** 211-221.

Prajapati, O.K. and Pandey, P.K. (2007) Isolation of copper resistant strain of a cyanobacterium *Nostoc calcicola* Breb. *Flora and Fauna. Internl. Res. J. Biol. Sc.*, **13** (2): 310-311.

Prasad, S.M., Singh J.B., Rai, L.C. and Kumar, H.D. (1991) Metal induced inhibition of photosynthetic electron transport chain of the cyanobacterium *Nostoc muscorum. FEMS Microbiol. Lett.*, **82:** 94-100.

Price, N.M. and Morell, F.M. (1990) Cadmium cobalt-substitution for zinc in a marine diatom. *Nature.*, **344:** 568-660.

Raboy, B., Padan, E. and Shilo, M. (1976) Heterotrophic capacities of *Plectonema boryanum. Arch. Microbiol.*, **110:** 77-85.

Rachlin, J.W., Jensen, T.E. and Warkentine, B.E. (1985) Morphometric analysis of the response of *Anabaena flos-aquae* and

Anabaena variabilis (Cyanophyceae) to selected concentrations of zinc. *Arch. Environ. Contam. Toxicol.*, **14:** 395-402.

Rachlin, J.W., Jensen, T.E. Baxter, M. and Jani, V. (1982) Utilization of morphometric analysis in evaluating response of *plectonema boryanum* (Cyanophycae) to exposure to eight heavy metals. *Arch. Environ. Contam. Toxicol.*, **11:** 323-333.

Rai, A.K. (1990) Biochemical characteristics of photosynthetic response to various external salinities in halo-tolerant and fresh water cyanobacteria. *FEMS Microbiol. Lett.*, **69:** 177-180.

Rai, A.K. (1997) Urea metabolism. In: *Cyanobacterial Nitrogen Metabolism and Environmental Biotechnology* (ed. A.K. Rai). *Narosa Publishing house, New Delhi, India. pp.* 73-87.

Rai, A.N. (1990) Handbook of symbiotic cyanobacteria. *CRC press, Boca Raton, Florida USA.*

Rai, A.N., Borthakur, M., Singh, S. and Bergman, B. (1989) *Anthoceros Nostoc* symbiosis: immuno-electronmicroscopic localization of *nitrogenase, glutamine synthetase,* phycoerythrin and *ribulose-1, 5-bisphosphate carboxylase / oxygenase* in the cyanobiont and the cultured (free-living) isolate *Nostoc* 7801. *J. Gen. Microbiol.*, **135:** 385-395.

Rai, A.N., Rowell, P. and Stewart, W.D.P. (1984) Evidence for an ammonium transport system in fee living and symbiotic cyanobacteria. *Arch. Microbiol.*, **137:** 241-247.

Rai, A.N., Soderback, E. and Bergman, B. (2000) Cyanobacterium plant symbiosis. *New Phytol.*, **147:** 449-481.

Rai, L.C. and Dey, R. (1980) Environmental effects on the toxicity of methyl mercuric chloride to *Chlorella mlyanis. Acta Hydrochim. ed. Hydrobiol.*, **8:** 319-327.

Rai, L.C. and Dubey, S.K. (1989) Impact of chromium and tin on a nitrogen-fixing cyanobacterium *Anabaena doliolum:* Interaction with bivalent cations. *Ecotoxicol Environ Saf.*, **17** (1)**:** 94-104.

Rai, L.C. and Raizada, M. (1985) Effect of nickel and silver ions on survival, growth, carbon fixation and nitrogen activity of *Nostoc*

muscorum: Regulation of toxicity by EDTA and Calcium. *J. Gen. Microbiol.*, **31:** 329-337.

Rai, L.C. and Raizada, M. (1986) Nickel induced stimulation of growth, heterocyst differentiation, $^{14}CO_2$-incorporation and *nitrogenase* activity in *Nostoc muscorum. New Phytol.*, **104:** 111-114.

Rai, L.C. and Raizada, M. (1987) Toxicity of nickel and silver to *Nostoc muscorum:* Interaction with ascorbic acid, glutathione and sulphur containing amino acids. *Ecotoxicol., Environ. Safty.*, **14:** 12-21.

Rai, L.C., Dubey, S.K., and Mallik, N. (1992) Influence of chromium on some physiological variables of *Anabaena doliolum*: Interaction with metabolic inhibitors. *Bimetals.*, **5:** 13-16.

Rai, L.C., Gaur, J.P. and Kumar, H.D. (1981) Phycology and heavy metal pollution. *Biol. Rev.*, **56:** 99-151.

Rai, L.C., Jensen, T.E. and Rachlin, J.W. (1990) Amorphometric and X-ray energy dispersive approach to monitoring pH-altered cadmium toxicity in *Anabaena flos-aquae., Arch. Environ. Contam. Toxicol.*, **19:** 479-487.

Rai, L.C., Raizada, M., Mallick, N., Husaini, Y., Singh, A.K. and Dubey, S.K. (1990) Effect of four heavy metals on the biology of *Nostoc muscorum. Bio met.*, **2** (4): 229-234.

Rai, L.C., Tyagi, B., Mallick, N. and Rai, P.K. (1998) Interactive effects of UV and heavy metals (Cu and Pb on nitrogen and phoswphorus metabolism of the Cyanobacterium *Anabaena doliolum. Environ. Exp. Bot.*, **39:** 221-231.

Rai, U.N. and Chandra, P. (1989) Removal of heavy metals from polluted water.

Rai, U.N., Tripathi, R.D., Singh, N., Kumar, A., Ali, M.B., Pal, A. and Singh, S.N. (2000) Bioamelioration of fly-ash by selected nitrogen fixing blue-green algae. *Bull. Environ. Contam. Toxicol.*, **64** (2): 294-301.

Raizada, M. and Rai, L.C. (1985) Metal induced inhibition of growth, heterocyst differentiation, carbon-fixation and *nitrogenase* activity *of*

Nostoc muscorum: Interaction with EDTA and calcium. *Microbiol. Lett.*, **30:** 153-161.

Ramamoorthy, S., Cheng, T.C. and Kushner (1992) Effect of microbial life stages on the fate of methyl mercury in natural waters. Bull. Environ. Contam. Toxicol., 29: 167-173.

Rana, B.C., Gopal, T. and Kumar, H.D. (1971) Studies on the biological effects of industrial wastes on the growth of algae. *Environ. Health.*, **13:** 138-143.

Rangasayatorn, N., Upatham, S.E., Kruatrache, M., Pokethityook, P. and Lanza, R.G. (2006) Phytoremediation and toxicity studies of Cd., *Environ. Pollut.*, **119:** 45-53.

Rashid, M.A. (1987) Role of humic acids of marine origin and their different molecular weight fractions in complexing di-and tri-valent metals. *Soil Sci.*, **111:** 298-306.

Ravendra, V., Scaria, J. and Verma, S.K. (2002) Application of mutant strains of cyanobacteria for Cd^{+2} removals. *Bull. Environ. Contam. Toxicol.*, **69**(5): 632-637.

Rayner, M.H. and Sadler, J.P. (1989) Cadmium accumulation and resistance mechanisms in bacteria. *Spec. Publ. Soc. Gen. Microbiol.*, **26:** 39-47.

Ren, L., Shi, O., Dai, J. and Ruth, B. (1998) Expression of the *metallothionein-I* gene conferring Cd resistance in a transgenic cyanobacterium. *FEMS Microbial Lett.*, **158:** 127-132.

Reynolds, C.S. and Walsby, A.E. (1987) Water blooms. *Biol. Rev.*, **50:** 437-481.

Rippka, R and Stanier, R.Y. (1978) The effect of anaerobiosis on *nitrogenase* synthesis and heterocyst development by Nostocacean cyanobacteria. *J. Gen. Microbiol.*, **105:** 83-94.

Rippka, R., Derulles, J., Waterbury, J.B., Herdman, M. and Stainer, R.Y. (1979) Generic assignments, strains, histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbial.*, **111:** 1-61.

References

Rippka, R., Derulles, J., Waterbury, J.B., Herdman, M., Robinson, P.K., Reeve, J.O. and Goulding, K.H. (1988) Kinetics of phosphorus uptake by immobilized *Chlorella. Biotechnol. Lett.,* **10:** 17-20.

Robinson, P.K., Reeve, J.O. and Goulding, K.H. (1988) Kinetics of phosphorus uptake by immobilized *Chlorella. Biotechnol. Lett.,* **10:** 17-20.

Robinson, P.K., Reeve, J.O. and Goulding, K.H. (1989) Phosphorus uptake kinetics of immobilized *Chlorella* in batch and continuous-flow culture. *Enzyme Microb. Technol.,* **11:** 590-596.

Ros, R., Cooke, D.T., Burden, R.S. and James, C.S. (1990) Effects of the herbicide MCPA, and the heavy metals, cadmium and nickel on the lipid composition, Mg^{+2}-*ATPase* activity and fluidity of plasmamembrane from rice *Oryza sativa* (C.V. Bahia) shoots. *J. Exp. Bot.,* **41:** 457-462.

Rowell, P., Enticott, S. and Stewart (1977) *Glutamine synthetase and nitrogenase* activity in the blue-green alga *Anabaena cylindrica. New Phytol.,* **79:** 41-54.

Roychowdhury, T. Uchino, T., Tokunaga, H. and Ando, M. (2002 a) Arsenic and other heavy metals in soils from an arsenic affected area of West Bengal, India. *Chemosphere.,* **49 :** 605-618.

Roychowdhury, T., Uchino, T., Tokunaga, H. and Ando, M. (2002 b) Survey of arsenic in food composites from an arsenic affected area of West Bengal, India. *Food Chem. Toxicol.*, **40:** 1611-1621.

Rubinson, J.F., Burgess, J.L., Corbin, J.L. and Dilworth (1985) *Nitrogenase* reactivity: Azide reduction. Biochemistry. **24:** 273-283.

Rueter, J.G., McCarty, J.J. and Carpenter, E.J. (1979) The toxic effect of copper on *Oscillatoria (Trichodesmium) theibanti.* Limnol. *Oceanogr.,* **24:** 558-562.

Sakagguchi, T., Tsuji, T., Nakajima, A. and Hosikoshi (1979). Accumulation of cadmium by green microalgae. *Eur. J. Appl. Microbiol.,* **8:** 207-215.

Sampaio, M.J.A.M. Rowell, P. and Stewart, W.D.P. (1979) Purification and some properties of *glutamine synthetase* from the

nitrogen-fixing cyanobacteria *Anabaena cylindrica* and a *Nostoc* sp. J. *Gen. Microbiol.*, **111:** 181-191.

Samson, G. and Papovic, R. (1990) Inhibitory effects of mercury on PS II photochemistry in *Dunaliella tertiolecta* under *in vivo* conditions. *J. Phytochem. Phytobiol. B. Biol.*, **5:** 303-310.

Say, P.J., Burrows and B.A. Whitton (1990) *Enteromorpha* as a monitor of heavy metals in estuaries. *Hydrobiologia.*, **195:** 119-126.

Schecher, W.D. and Driscoll, C.T. (1985 a) Interaction of copper and lead with *Nostoc muscorum. Water, Air, Soil Pollut.*, **24:** 85-101.

Schecher, W.D. and Driscoll, C.T. (1985) Water Air Soil Pollut., **24:** 85-101.

Scherer, S. Almon, H. and Boger, P. (1988) Interaction of photosynthesis, respiration and nitrogen fixation in cyanobacteria. *Photosynth. Res.*, **15:** 94-114.

Schnabl, H., Youngman, R.J. and Zimmermann, U. (1983) Maintenance of plant cell membrane integrity and function by the immobilization of protoplast in alginate matrices. *Planta., 158: 392-397.*

Schnitzer, M. and Khan, S.U. (1978) Soil Organic Matter. *Elsevier, New York.*, **22:** 319-327.

Schoof, J.W. (1975) Pre-Cambrian paleobiology: problems and perspectives. *Ann. Rev. Earth Planet Sci.*, **3:** 213-249.

Schoof, J.W. (2000) The fossil record: Tracing of the roots of the cyanobacterial lineage, In: *The Ecology of Cyanobacteria* (eds. B.A. Whitton and M. Potts). *Kluwer Academic Publishers, Dordrecht, The Netherlands., pp.* 13-35.

Scott, J.A., Slage, D.A. and Palmer, S.J. (1988) Metal immobilization by microbial capsular coatings. *Biorecovery.* **10:** 51-58.

Shapiro, B.M. and Stadtman, E.R. (1970) *Glutamine synthetase* (*Escherechia coli*). In: *Methods in Microbiology* (ed. H. Tabor and C.W. Tabor). *Acadmic Press, London and New York,* **17:** 910-922.

Shehata, F.H.A. and Whitton, B.A. (1982) Zinc tolerance in strains of Blue-green algae, *Anacystis nidulans*. *Br. Phycol. J.,* **17:** 5-12.

Shi, D.J., Brouers, M., Hall, D.O. and Robins, R.J. (1987) The effects of immobilization on the biochemical, physiochemical and morphological features of *Anabaena azollae. Planta* (*Berl.*)., **172:** 298-308.

Shieh, Y.J. and Barber, J. (1973) Uptake of mercury by *Chlorella* and its effect on potassium regulation. *Planta,* **109:** 49-60.

Shilo, M. (1982) Diversity of the photosynthetic prokaryotes. In: *On the origins of chloroplasts* (ed. J.A. Schiff). *Elsevier, North Holland., pp.* 9-26.

Shioi, Y., Tami, H. and Sasa, T. (1973) Inhibition of photosystem II in the green algae *Ankistrodesmus calcatus* by copper. *Plant Physiol.,* **44:** 434-438.

Shioi, Y., Tami, H. and Sasa, T. (1978) Effect of copper on photosynthetic electron transport system in Spinach chloroplast. *Plant Physiol.,* **19:** 203-209.

Shubert, L.E. (1984) Algae as ecological indicators. *Academic Press, London.*

Sidler, W.A. (1994) Phycobilisome and phycobiliprotein structures. In: *The Molecular Biology of Cyanobacteria.* (ed. D.A. Bryant), *Kluwer Academic Publishers, Dordrecht, The Netherlands., pp.* 139-216.

Silver, S. (1977) Calcium transport in microorganism and Minerals (ed. E.D. Weinberg). *Dekker, New York.*

Silver, S. and Keach, D. (1982) Energy dependent arsenate efflux: The mechanism of plasmid mediated resistance. Proc. *Natl. Acad. Sci. USA.,* **79:** 6114-6118.

Simon, R.D. (1973) Measurement of the cyanophycean granule polypeptide contained in the blue-green alga *Anabaena cylindrica. J. Bacteriol.,* **114:** 1213-1216.

Simon, R.D. (1977) Sporulation in the filamentous cyanobacterium *Anobaena cylindrica.* The course of spore formation. *Arch. Microbiol.,* **111:** 283-288.

Simon, R.D. and Weathers, P. (1976) Determination of the structure of the novel polypeptide containing aspartic acid and arginine which is found in cyanobacteria. *Biochem. Biophys. Acta.,* **20:** 165-176.

Simpson, F.B. and Neilands J.B. (1976) Siderochromes in cyanophyceae: Isolation and characterization of schizokinen from *Anabaena cylindrica. J. Phycol.,* **12:** 44-48.

Sinclair, C. and Whitton, B.A. (1977a) Influence of nutrient deficiency on hair formation in the Rivulariacae. *J. Br. Phycol.,* **12:** 297-313.

Sinclair, C. and Whitton, B.A. (1977b) Influence of nitrogen source on morphology of Rivulariaceae (Cyanophyta). *J. Phycol.,* **13:** 335-340.

Singh, B.B., Chauhan, V.S., Gour, R.K., Singh, S. and Bisen, P.S. (1999) Heterocysts: Present status and future prospects. *J. Ind. Bot. Sci.,* **78:** 25-32.

Singh, B, Chauhan, V.S., Singh, S. and Bisen, P.S. (2001) Isolation and partial characterization of *Het⁻ Fix⁻* mutant strain of diazotrophic cyanobacterium *Anabaena variabilis* showing chromatic adaptation. *Curr. Microbiol.,* **43:** 265-270.

Singh, B.B., Pandey, P.K., Singh, S. and Bisen, P.S. (1996) Regulation of nitrate uptake and nitrite efflux in the cyanobacterium *Nostoc* MAC. *J. Basic Microbiol.,* **36:** 433-438.

Singh, B.B., Pandey, P.K., Singh, S. and Bisen, P.S. (1997) Calcium induced physiological and biochemical changes in the cyanobacterium *Nostoc* MAC I. *J. Exp. Biol.,* **35:** 881-885.

Singh, C.B. (1987) Physiological studies on the cyanobacterium *Nostoc calcicola* under mercury stress. *J. Plant Physiol.,* **67:** 67-99.

Singh, C.B. and Singh, S.P. (1987) Effect of mercury on photosynthesis in *Nostoc calcicola*: Role of ATP and interacting heavy metal ions. *J. Plant Physiol.,* **129:** 49-58.

Singh, C.B., Verma, S.K., and Singh, S.P. (1987) Impact of heavy metals on *glutamine synthetase* and *nitrogenase* activity in *Nostoc calcicola. J. Gen. Appl. Microbiol.*, **33:** 87-91.

Singh, D.P. (1985) Cu^{2+} transport in the unicellular cyanobacterium, *Anacystis nidulans. J. Gen. Appl. Microbiol.*, **31:** 277-284.

Singh, D.P. and Singh, S.P. (1987) Action of heavy metals on Hill activity and O_2-evolution in *Anacystis nidulans. Plant Physiol.*, **83:** 12-14.

Singh, D.P., Khare, P. and Bisen, P.S. (1989) Effect of Ni^{2+}, Hg^{+2} and Cu^{+2} on growth, oxygen evolution and photosynthetic electron transport in *Cylindrospermum* PCC 7942. *J. Plant Physiol.*, **134:** 406-412.

Singh, H.N. and Singh, H.N. (1978) An Azide-resistant mutant of the blue-green algae *Nostoc muscorum* producing heterocysts and *nitrogenase* in the presence of fixed inorganic nitrogen source. *Arch. Microbiol.*, **14:** 1341-1346.

Singh, H.N., Stewart, W.D.P. and Singh, H.N. (1975) Transfer of nitrogen fixing *nif* gene in blue-green algae, *Nostoc muscorum. Biochem. Biophys. Common.*, **62:** 62-69.

Singh, N., Asthana, K.R., Kayastha, M.A., Pandey, S., Chaudhary, K.A. (1999) Thiol and exopolysaccharide production in a cyanobacterium under heavy metal stress. *Process Biochem.*, **35:** 63-68.

Singh, P.K. (1978) Nitrogen economy of rice soils in relation to nitrogen fixation by blue-green algae and *azolla.* In: *National Symposium on increasing rice yield in Khariff,* Cuttak, Central Rice Research Institute, Cuttak, India.

Singh, R.N. (1939) An investigation into algal flora of paddy soils in the United Provinces. *Ind. J. Agric. Sci.*, **12:** 743-756.

Singh, R.N. (1950) Reclamation of usar-lands in India through blue-green algae. *Nature (London).*, **165:** 325-326.

References

Singh, R.N. (1961) Role of blue-green algae in nitrogen economy of Indian agriculture. Indian Council if Agricultural Research, New Delhi, India. pp. 175.

Singh, R.N. (1972) Physiology and Biochemistry of nitrogen-fixation by blue-green algae. *Final technical report* (1967-72), Publication Banaras Hindu University, India, *pp.* 66-67.

Singh, S. and Bisen, P.S. (1993) Evidence for the role of intracellular glutamine level in the regulation of glutamine uptake in the cyanobacterium, *Anabaena* 7120. *Curr. Microbiol.,* **29:** 319-322.

Singh, S. and Bisen, P.S. (1994) Role of *glutamine synthetase* activity in the urea inhibition of heterocyst and *nitrogenase* formation in the cyanobacterium *Anabaena cycadeae. J. Basic Microbiol.,* **34:** 191-195.

Singh, S., Singh, B.B. and Bisen, P.S. (1995) of ammonium assimilation in the urea inhibition of *nitrogenase* activity in the cultured cyanobiont *Nostoc* ANTH. *Ind. J. Exp. Biol.,* **33:** 94-96.

Singh, S.P. and Pandey, A.K. (1981a) Cadmium toxicity in a cyanobacterium: Effects of modifying factors. *Environ. Exp. Bot.,* **21:** 257-265.

Singh, S.P. and Pandey, A.K. (1981b) Heavy metal interaction studies with blue-green algae. *Acta Bot. Indica.,* **9:** 290-296.

Singh, S.P. and Pandey, A.K. (1982) Cadmium mediated resistance to heavy metals and antibiotics in a cyanobacterium. *Molec. Gen. Gent.,* **187:** 240-243.

Singh, S.P. and Yadava, V. (1984) Cadmium induced inhibition of ammonium and phosphate uptake in *Anacystis nidulans:* Interaction with other divalent cations. *J. Gen. Appl. Microbiol.,* **31:** 39-48.

Singh, S.P. and Yadava, V. (1985) Cadmium uptake in *Anacystis nidulans:* effect of modifying factor: *J. Gen. Appl. Microbiol.,* **31:** 39-48.

Singh, S.P. and Yadava, V. (1986) Cadmium tolerance in the cyanobacteriaum *Anacystis nidulans. Biol. Zentralbl.,* **105:** 539-542.

References

Singh, S.P., Srivastava, S.C. and Pandey, K.D. (1990) Photo-production of hydrogen by a non-sulphur bacterium isolated from root zones of water fern *Azolla pinnata. Int. J. Hydrogen Energy.*, **15:** 403-406.

Singh, S.P., Verma, S.K., Singh, R.K. and Pandey, P.K. (1989) Copper uptake by a free and immobilized cyanobacterium. *FEMS Microbiol. Lett.*, **60:** 193-196.

Sivonen, K., Lyra, C., Hantula, J., Vainio, E., Rapala, J. and Rouhiainen, L. (1997) Characterization of cyanobacteria by SDS-PAGE of whole cell proteins and PCR/RFLP of the 16S *rRNA* genes. *Arch. Microbiol.*, **168:** 176-184.

Skowronski, T. and Czernas, K. (1985) Growth of *Stichococcus bacillaris* on media containing Cd or Zn. *Acta Hydrodiol.*, **25:** 401-408.

Smith, A.J. (1982) Modern of cyanobacterial carbon metabolism. In: The Biology of Cyanobacteria. (eds. N.G. Carr and B.A. Whitton) *Blackwell Scientific Publications, Oxford, London., pp.* 4-7.

Smith, M.J., Shoolery, J.N., Schwyn, B., Holden, I. and Neiland, J.B. (1985) Rhizobactin, a structurally novel siderophore from *Rhizobium meliloti. J. Am. Chem. Soc.*, **107:** 1739-1743.

Smith, R.J., Hobson, S. and Ellis, I.R. (1987) The effect of abscisic acid on calcium-mediated regulation of heterocyst frequency and *nitrogenase* activity in *Nostoc* 6720. *New Phytol.*, **5:** 543-549.

Snell, F.D. and Snell, C.T. (1949) Colorimetric Methods of Analysis. *3rd Edition Van Nostrand, New York. pp.*, 804-805.

Snow, G.A. (1954) J. Chem. Soc., 2588.

Sorentino, C. (1979) The effects of heavy metal on phytoplankton: *A review. Phycos.*, **18:** 149-161.

Sprent, J.I. and Sprent, P. (1990) Organisms that fix nitrogen. In: *Nitrogen-fixing organisms, pure and applied aspects. Cambridge University Press, London. pp.*, 1-15.

Stacey, G., Tabita, F.R. and Baalen, C.V. (1977) Nitrogen and ammonia assimilation in the cyanobacterium: Purification of *glutamine synthetase* from *Anabaena* sp. strain CA. *J. Bacteriol.,* **132:** 596-603.

Stal, L.J. (2000) Cyanobacterial mats and stromalites. In: *The Ecology of Cyanobacteria.* (eds. B.A. Whitton and M. Potts.). *Kluwer Academic Publishers, Dordrecht, London.,* 61-120.

Stanier, R.Y. (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.,* **111:** 1-61.

Stanier, R.Y. and Cohen-Bazire, G. (1977) Phototrophic prokaryotes: The Cyanobacteria. *Ann Rev Microbiol.,* **31:** 225-274.

Starodub, M.E., Wong, P.T.S., Mayfield, C.I. and Chau, Y.K. (1987) Influence of complexation and pH on individual and combined heavy metal toxicity to a fresh water green algae. *Can. J. Fish Aquat. Sci.,* **44:** 1173-1180.

Steeman-Nielsen, E. and Kamp-Nielsen L. (1970) Influence of deleterious concentrations of copper on the growth of *Chlorella pyrenoidosa. Plant Physiol.,* **23:** 828-840.

Stevenson, F.J. (1976) Stability constants of Cu^{2+}, Pb^{2+} and Cd^{2+} complexes with humic acids. *Soil Sci. Soc. Am. Proc.,* **40:** 665-672.

Stevens, S.E., Paone, D.A.M. and Balkwill, D.L. (1981) Accumulation of cyanophycean granules as a result of phosphate limitation in *Agmenllum quadruplicatum. Plant Physiol.,* **67:** 716-719.

Stewart, W.D.P. (1973) Nitrogen-fixation by photosynthetic microorganisms. *Ann. Rev. Microbiol.,* **27:** 283-316.

Stewart, W.D.P. (1980) Some aspects of structure and function in nitrogen fixing cyanobacteria. *Ann. Rev. Microbiol.,* **34:** 497-536.

Stewart, W.D.P. and Lex, M. (1970) *Nitrogenase* activity in the blue-green algae *Plectonema boryanum* strain 594. *Arch. Microbiol.,* **73:** 250-260.

Stewart, W.D.P. and Rowell, P. (1986) Biochemistry and physiology of nitrogen-fixation with particular emphasis on nitrogen-fixing phototrophs. *Plant Soil.*, **90:** 167-191.

Stewart, W.D.P. and Singh, H.N. (1975) Transfer of nitrogen-fixing (*nif*) genes in the blue-green algae *Nostoc calcicola., Biochem. Biophys. Res. Commun.*, **62:** 62-69.

Stewart, W.D.P., Fitzgerald, G.P. and Burris, R.H. (1967) *In situ* studies on N_2-fixation using acetylene reduction technique. *Proc. Natl. Acad. Sci. USA.*, **58:** 2071-2078.

Stewart, W.D.P., Fitzgerald, G.P. and Burris, R.H. (1968) Acetylene reduction by nitrogen-fixing blue-green algae. *Arch. Microbiol.*, **62:** 336-348.

Stewart, W.D.P., Fitzgerald, G.P. and Burris, R.H. (1970) Acetylene reduction assay for determination of phosphorus availability in Wisconsin Lakes. *Proc. Natn. Acad. Sci., U.S.A.*, **66:** 1104-1111.

Stewart, W.D.P., Haystead, A. and Dharmawardene, M.W.N. (1975) Nitrogen assimilation and metabolism in blue-green algae. In: *Nitrogen Fixation in free living microorganisms* (ed. W.D.P.Stewart). *Cambridge Univ. Press, Cambridge, pp.* 129-159.

Stewart, W.D.P., Rowell, P. and Rai, A.N. (1983) Cyanobacteria-eukaryotic plant symbiosis. *Ann. Microbiol.*, **134:** 205-228.

Stewart, W.D.P., Rowell, P., Kerby, N.W., Reed, R.H. and Machray, G.C. (1987) N_2-fixing cyanobacteria and their potential applications. Philosophical Transactions of the Royal society of London B., **317:** 245-258.

Stockes, P.M. (1975) Uptake and accumulation of copper and nickel by metal tolerant strains of *Scenedemus quadricauda. Verh. Int. Verein. Limnol.*, **19 :** 2128-2137.

Stokes, P.M. (1979) Copper accumulations in fresh-water biota. (ed. J.O. Nriagu), *Copper in the environment. V.L. Intersc.*, pp. 357.

Stockes, P.M. (1983) Response of freshwater algae to metals. In: *Progress in Phycological Research* (eds. F.E. Round and D.J. Chapman). *Elsevier Sci. Publishers, Amsterdam.*, **2:** 87-112.

References

Stokes, P.M. and Hutchinhon, T.C. (1975) Copper toxicity to phytoplankton as affected by organic ligands, other cations and inherent tolerance of algal to copper. In: *Symposium of the International Joint Commission on the Great Lakes* (eds. R.W. Andrew, P.V. Hodson and D.E. Konasewich). *Windsor, Canada, pp.* 159-185.

Stratton, G.W. (1985) Interaction effects of mercury-pesticide combinations towards a cyanobacterium. *Bull. Environ. Contam. Toxiciol.,* **34:** 676-683.

Stratton, G.W. and Corke, C.T. (1979a) The effect of cadmium ion on the growth, photosynthesis and *nitrogenase* activity of *Anabaena inaequalis. Chemosphere.,* **5:** 277-282.

Stratton, G.W. and Corke, C.T. (1979b) The effect of nickel ion on the growth, photosynthesis and *nitrogenase* activity of *Anabaena inaequalis. Can. J. Microbiol .,* **25:** 1094-1099.

Stratton, G.W. and Corke, C.T. (1979c) The effect of mercuric cadmium and nickel ion combinations on the blue-green algae. *Chemosphere.,* **10:** 731-740.

Stratton, G.W., Huber, A.L. and Corke, C.T. (1979) The effect of mercuric ion on the growth, photosynthesis and *nitrogenase* activity of *Anabaena inaequalis. Appl. Environ. Microbiol.* , **38:** 537-543.

Subramanian, G. and Uma, L. (1996) Cyanobacteria in pollution control. *J. Sci. Indus. Res.* **55:** 685-692.

Subramanian, G., Shekhar, S. and Sampoornam, S. (1994) Biodegradation and utilization of organophosphorus pesticides by cyanobacteria. *Int. J. Biodeter Biodegra.,* **33:** 129-143.

Sunda, W.G. and Lewis, J.A.M. (1978) Effect of complexation by natural organic ligands on the toxicity of copper to a unicellular alga *Monochysis lutheri. Limnol. Oceaogr.,* **23:** 870-876.

Suresh Babu, G., Farooq, M., Singh, J., Vishwanathan, P.N., Joshi, P.C. and Hans, R.K. (2001) Metabolic alterations due to exposure of Lindane in Basmati rice, *Oryzae sativa* Seedlings. *Pollution Research.,* **19:** 523-528.

References

Sutherland, J.M., Herdman, M. and Stewart, W.D.P. (1979) Akinetes of the cyanobacterium *Nostoc* PCC 7524: macromolecular composition, structure and control of differentiation. *J. Gen. Microbiol.*, **115:** 273-287.

Syrett, P.J. (1988) Uptake and utilization of nitrogen compounds. In: *Biochemistry of the algae and cyanobacteria.* (eds. L.J. Rogers and J.R. Gallon). *Clarendon Press, Oxford.*, **28:** 23-40.

Takamura, N., Kasai, F. and Watanabe, M.M. (1992) Effects of Cu, Cd and Zn on photosynthesis of freshwater benthic algae. *J. Appl. Phycol.*, **12:** 39-52.

Tandeau de Marsac N. and Houmard, J. (1993) Adaptation of cyanobacteria to environmental stimuli: new steps towards molecular mechanisms. *FEMS Microbiol. Rev.*, **104:** 119-190.

Taneja, L. and Fatma, T. (2000) Studies on potential use of Cyanobacterium westidlopsis for bioremediation of Cu. Environental Stress, indication, mitigation and eco-conservation, edited by Yunus *et al., Kluwer Academic Publishers., The Netherlands.*

Terry, A.P. and Stere, W. (2000) Biosorption of Cd and Cu contaminated water by *Scenedesmus obundans. Chemosphere.*, **47:** 249-255.

Thiel, T. (1990) Protein turnover and heterocyst differentiation in the cyanobacterium *Anabaena variabilis. J. Phycol.*, **26:** 50-54.

Thiel, T. (1994) Genetic analysis of cyanobacteia. In: *The Molecular Biology of Cyanobacteia* (ed. D.A. Bryant). *Kluwer Academic Publishers., USA.*

Thiel, T. (1996) Isolation and characterization of the *vnfEN* genes of the cyanobacterium *Anabaena variabilis. J. Bacteriol.*, **178:** 4493-4499.

Thomas, G.P., Windhovel, U.R., Peter, B.S. and Sandmann, G. (1999) Protection of photosynthesis against Ultraviolet radiations by carotenoids in transformation of the cyanobacteria *Synechoccocus. Plant Physiol.*, **120:** 599-604.

Thomas, J., Meeks, J.C., Wolk, C.P., Shaffer, P.W., Austin, S.M. and Chen, W.S. (1977) Formation of glutamine from (^{13}N) ammonia, (^{13}N) dinitrogen and (^{14}C) glutamate by heterocysts isolated from *Anabaena cylindrica. J. Bacteriol.*, **129:** 1545-1555.

Thomas, J., Wolk, C.P., Shaffer, P. W., Austin, S.M. and Galonsky, A. (1975) The initial organic products of fixation of ^{13}N-labelled nitrogen gas by the blue green alga *Anabaena cylindrica. Biochem Biophys. Res. Commun.*, **67:** 501-507.

Thomas, S.B., Neild, Jor and Barber, J. (2001) Iron deficiency induces the formation of an antenna ring around trimeric photosystem-I in cyanobacteria. *Nature.*, **412:** 743-746.

Thompson, S.L., Manning, F.C.R., McCall, S.M. (2000) Comparison of morality of Cr^{+3} and Cr^{+6} to cyanobacteria. *Bull. Environ. Contam. Toxicol.*, **69**: 286-293.

Tien, J.C. (2002). Biosorption of metal ions by fresh water algae with different surface characteristics. *Biochem.*, **38:** 605-613.

Ting, Y.P., Lawson, F. and Prince, G. (1989) Uptake of cadmium and zinc by the algae *Chlorella vulgaris*: Part-I. Individual iron species. *Biotechnol. and Bioeng.*, **34:** 990-995.

Ting, Y.P., Lawson, F. and Prince, I.G. (1990) The uptake of heavy metal ions by algae. *Austr. J. of Biotechnol.*, **4:** 197-200.

Ting, Y.P., Lawson, F. and Prince, G. (1991) Uptake of cadmium and zinc by the algae *Chlorella vulgaris*: mutlti-ions situation. *Biotechnol. and Bioeng.*, **37:** 605-613.

Tiwari, G.L. (1972) A study of the blue-green algae from paddy field soils of India. *Hydrobiologia.*, **39:** 335-350.

Tiwari, O.N., Dolly, W.D., Prasana, R., Shukla, H.M., Singh, P.K. and Tiwari, G.L. (2000) Growth and nitrogen fixation by non-heterocystous filamentous cyanobacteria of rice fields of Uttar Pradesh, India. *Philip. J. Sci.* **129:** 101-107.

Tohoyama, H. and Murayama, T. (1977) Isolation and some characteristics of cadmium resistance yeasts. *Agric. Biol. Chem.*, **41:** 1523-1524.

Torriani, A. (1960) Influence of inorganic phosphate in the formation of phosphate by *Eschechia coli. Biochem. Biophy. Acta,* **38:** 460-479.

Trebst, A. (1974). Energy conservation in photosynthetic electron transport of chloroplasts. *Ann. Rev. Plant Physiol.,* **25:** 423-458.

Trehan, K. and Maneesha, N. (1991) Effects of lead on *nitrogenase* and enzymes of nitrogen assimilation in a cyanobacterium *Nostoc muscorum. Ind. J. Exp.Bot.,* **29:** 1116-1119.

Trollope, D.R. and Evans, B. (1976) Concentrations of copper, iron, lead, nickel and zinc in freshwater algal blooms. *Environ. Pollut.,* **11:**109-116.

Turner, I.S., Robinson, N.J. (1995) Cyanobacterial *Metallothioneins*: Biochemistry and Molecular Genetics, *J. Ind. Micobiol.,* **14:** 119-129.

Tyler, G. (1981) Heavy metals in soil biology and biochemistry. In: *Soil Biochemistry* (eds. A.E. Paul and J.N. Ladd). *Marcel Dekker, New York, pp.* 371-414.

Tynecka, Z., Gos, Z. and Zajac, J. (1981a) Reduced cadmium transport determined by a resistance plasmid in *Staphylococcus aureus. J. Bacteriol.,* **147:** 305-312.

Tynecka, Z., Gos, Z. and Zajac, J. (1981b) Energy-dependent efflux of cadmium coded by a plasmid resistance determined in *Staphylococcus aureus. J. Bacteriol.,* **147:** 313-319.

Tynecka, Z., Skwarek, T. and Malam, A. (1990) Anaerobic ^{109}Cd accumulation by cadmium resistant and sensitive *Staphylococcus aureus. FEMS Microbiol., Lett.,* **69:** 159-164.

Tyneeva, Z., Zos and Zazae, J. (1982) Reduced cadmium transport determined by resistant plasmid in *Staphylococcus aureus. J. Bacteriol.,* **147:** 305-312.

Uribe, E.G. and Stark, B. (1982) Inhibition of photosynthetic energy conversion by cupic ions: Evidance for Cu^{2+} coupling factor-1 inactivation. *Plant Physiol.,* **69:** 1040-1045.

Vallee, B.L. (1959) Metal and enzyme interactions: Correlation of composition, function and structure. *Physiol. Rev.*, **39:** 443-447.

Vallee, B.L. and Ulmer, D.D. (1972) Biological effects of mercury cadmium and lead. *Ann. Rev. Biochem.*, **41:** 91-128.

Van den Berg, C.M.C., Wong, P.T.S. and Chau, Y.K. (1987) Measurement of complexing material excreted from algae and their ability to ameliorate copper toxicity. *J. Fish. Res. Bd. Can.*, **36:** 901-905.

Vasseur, P., Bois, F., Ferard, J.F., Rast, C. and Larbaight, G. (1986) Influence of physiochemical parameters on the microbial toxicity test response. *Tox. Assess.*, **1:** 283-300.

Vasseur, P., Pandard., P. and Burnel, D. (1988) Influence of some experimental factors on metal toxicity to *Selenastrum capricornutum. Tox. Assess.*, **3:** 331-343.

Verma, S.K. (1988) Uptake and toxicity of copper in the diazotrophic cyanobacterium *Nostoc calcicola* Breb. Ph.D. Thesis, Centre of Advanced Study in Botany, Banares Hindu University, Varanasi, India.

Verma, S.K. and Singh, S.P. (1990) Factors regulating copper uptake in a cyanobacterium. *Curr. Microbiol.*, **21:** 33-37.

Verma, S.K. and S.P. Singh (1991) Evidence for energy-dependent copper efflux as a mechanism of Cu^{+2} -resistance in the cyanobacterium *Nostoc calcicola., FEMS Microbiol. Lett.*, **84:** 291-294.

Venkataraman, G.S. (1961) The role of blue-green algae in agriculture. *Sci Cult.*, **27:** 9-13.

Venkataraman, G.S. (1979) Algal inoculation of rice fields. In: *Nitrogen and Rice,* International Rice Research Institute, Los Banos, Philippines. *pp.* 311-321.

Venkataraman, G.S. and Shanmugasundarum, S. (1982) Algal biofertilizer technology of rice, D.B.T. Centre for BGA biofertilizer, Madurai Kamraj University, Madurai-25021.

References

Venkataraman, G.S. and Shanmugasundarum, S. (1992) Utilization of atmospheric nitrogen by algae. Madurai Kamraj University, Madurai-25021.

Vincenzini, M., Balloni, W., Mannelli, D. and Florenzano, G. (1981) A bioreactor for continuous treatment of waste-waters with immobilized cells of photosynthetic bacteria. *Experimentia.*, **37:** 710-712.

Visviki, I. and Rachlin, J.W. (1991) The toxic action and interactions of copper and cadmium to the marine algae, *Dunaliella minuta* in both acute and chronic exposure. *Arch. Environ. Contam. Toxicol.*, **2:** 271-275.

Volesky, B., and Hollen Z.R. (1995) Biosorption of heavy metals. *Biotechnol. prog.*, **11:** 235-250.

Vymazal, J. (1987) Zinc uptake by *Cladophora glomerata. Hydrobiologia.*, **148:** 97-101.

Vymazal, J. (1990) Uptake of lead chromium, cadmium and cobalt by *Cladophora glomerata. Bull. Environ. Contam. Toxicol.*, **44:** 468-472.

Walsby, A.E. (1974) The extra-cellular products of *Anabaena cylindrica* Lemm. I. Isolation of a macromolecular pigment peptide complex and other components. *Br. Phycol. J.*, **9:** 371-381.

Wang, H. and Wood, J.M. (1984) Bioaccumulation of nickel by algae. *Environ. Sci. Tech.*, **18:** 106-109.

Werkowse, B.T. and Helena, K.D. (1985) Toxicity of zinc, cadmium, lead, copper and their mixture for *Chlorella pyrenoidosa. Acta Hydrobiol.*, **25:** 389-400.

Whitton, B.A. (1965) Extracellular products of blue-green algae. *J. Gen. Microbiol.*, **40:** 1-11.

Whitton, B.A. (1970) Toxicity of heavy metals to algae: A review. *Phykos.*, **9:** 116-125.

Whitton, B.A. (1984) Algae as monitor of heavy metals in freshwater. In: *Algae as ecological indicators* (ed. L.E. Shubert). *Academic Press, London.*, **11:** 257-280.

Whitton B.A. (1992) Diversity, ecology and taxonomy of the cyanobacteria. In: *Photosynthetic Prokaryotes* (eds. N.H. Mann & N.G. Carr). *Plenum Press, New York.* pp. 1-51.

Whitton B.A. (2000) Soils and rice fields. In: *The Ecology of Cyanobacteria* (eds. B.A. Whitton & M. Potts). *Kluwer Academic Publishers, Dordrecht, The Netherlands.* pp. 233-255.

Whitton, B.A. and Potts, M. (2000) Introduction to cyanobacteria, In: *The Ecology of Cyanobacteria* (eds. B.A. Whitton & M. Potts). *Kluwer Academic Publishers, Dordrecht, The Netherlands.* pp. 1-11.

Whitton, B.A. and Potts, S.P. and Malcolm, H.W. (2000) Introduction to the cyanobacteria: (eds. B.A. Whitton and M. Potts) *The Ecology of Cyanobacteria.,* pp. 125-137.

Whitton, B.A., Burrows, I.J. and Kelly, M.G. (1989) Use of *Cladophora glomerata* to monitor heavy metals in rivers. *J. Appl. Phycol.,* **1:** 293-299.

Wikfors, G.H. and Ukeles, R. (1982) Growth and adaptation of esturine unicellular algae in media with excess copper, cadmium or zinc and effects of metal-contaminated algal food on *Crassostrea virginica* larvae. *Mar. Ecol. Prog. Ser.,* **7:** 191-206.

Wilde, E.W. and Benemann, J.R. (1993) Bioremoval of heavy meals by the use of microalgae. *Biotech. Adv.* **11:** 781-792.

Wildon, D.C. and Mercer, F.V. (1963) The ultra-structure of the heterocyst and akinete of the blue-green algae. *Arch. Microbiol.,* **47:** 19-31.

Wilikinson, S.C., Goulding, K.H. and Robinson, P.K. (1989) Mercury accumulation and volatilization in immobilized algal cell systems. *Biotechnol. Lett.,* **11:** 861-864.

Wilson, D.E. (1978) An equilibrium model describing the influence of humic materials on the speciation of Cu^{+2}, Zn^{2+} and Mn^{+2} in fresh-waters. *Limnol. Oceanogr.,* **23:** 499-507.

Winner, R.W. (1985) Bioaccumulation and toxicity of copper as affected by interactions between humic acid and water hardness. *Water Res.*, **19:** 449-455.

Woese, C.R. (1987) Bacterial evolution. *Microbiol. Rev.*, **51:** 221-271.

Woese, C.R. and Fox, G. E. (1977) Phylogenctic structure of the prokaryotic domain: The primary kingdoms. *Proc. Natl. Acad. Sci., USA.*, **74:** 5088-5090.

Wolk, C.P. (1965) Heterocyst germination under defined conditions. *Nature (London).*, **205:** 201-202.

WoIk, C.P. (1973) Physiology and cytological chemistry of blue-green algae. *Bacteriol. Rev.*, **37:** 32-101.

Wolk, C.P. (1982) Heterocyst In: The Biology of cyanobacteria. (eds., N.G. Carr and B.A. Whitton) *Blackwell Sci. Publ. Oxford., pp.* 359.

Wolk, C.P. (1991) Genetic analysis of cyanobacterial development. *Curr. Opin. Gene Dev.*, **1:** 336-341.

Wolk, C. P., Cai, Y., Cardemil, L., Flores, E., Hohn, B., Murry, M., schmetterer, G., Schrautemeier, B. and Wilson, R. (1988) Isolation and complementation of mutants of *Anabaena* strain PCC 7120 unable to grow aerobically on dinitrogen. *J. Bacteriol.*, **170:** 1239-1244.

Wolk, C.P., Ernst, A. and Elhai, J. (1994) Heterocyst metabolism and development. In: *The Molecular Biology of Cyanobacteria* (ed. D. A. Bryant). *Kluwer Academic Publishers, Dordrecht, The Netherlands.*, **1:** 769-823.

Wood, J.M. (1974) Biological cycles for toxic elements in the environment. *Science.*, ***183:*** *1049-1052.*

Wood, J.M. and Wang, H.K. (1983) Microbial resistance to heavy metal. *Environ., Sci. Technol.*, **17:** 582-590.

Wood, N.B. and Haselkorn, R. (1979) *Proteinase* activity during heterocyst differentiation in nitrogen fixing cyanobacteria. In: *Limited Proteolysis in Microorganism* (eds. G.N. Cohen and H. Holzer). *U.S. DHEW Publication No. (N.I.H.) 79-1591, Bethesda Md., pp.* 159-166.

Wood, P., Peat, A. and Whitton, B.A. (1986) Influence of phosphorus status on fine structure of the cyanobacterium *Calothix parietina. Cytobios,* **47:** 89-99.

Wong, T.C., Welssman, J.C., Ramesh, G., Varadarajan, R. and Beneram, J.R. (1998) Heavy metal binding and removal by *Phoromidium. Bull. Environ. Contam. Toxicol.,* **60:** 739-744.

Wurtsbaugh, W.A. and Horne, A.J. (1982) Effects of copper on nitrogen-fixation and growth of blue-green algae in natural plankton associations. *Can. J. Fish. Aquat. Sci.,* **39:** 1636-1641.

XianKong, Z., Tabia, F.R. and Van Ballen, C. (1984) Nickel control of hydrogen production and uptake in *Anabaena species. Gen. Microbiol.,* **30:** 1815-1818.

Yadava V. (1984) Cadmium toxicity in the blue-green alga *Anacystis nidulans.* Ph.D. Thesis. Centre of Advanced study in Botany, Banaras Hindu University, Varanasi, India.

Yamamoto, Y. (1975) Effect of dessication on the germination of akinetes of *Anabaena cylindrica. Plant Cell Physiol.,* **16:** 749-752.

Yanagimoto, M., Saitoh, H. and Kakimoto, N. (1983) Alkaline shift effect on the uptake of germanium by algae, *Chlorella ellipsoideae, Oscillatoria* sp. and *spirulina platensis. J. Ferment. Technol.,* **61:** 233-236.

Yan-Chu, H. (1994) Arsenic distribution in soils. In: *Arsenic in the environment part I: Cycling and characterization.* (Ed. J.O. Nriagu), *John Wiley and Sons, Inc. New York., pp. 17-51.*

Yoon, H. S. and Golden, J. W. (1998) Heterocyst pattern formation controlled by a diffusible peptide. *Science (New York).,* **282:** 935-938.

Yoon, H. S. and Golden, J. W. (2001) Products of nitrogen fixation control heterocyst pattern. *J. Bacteriol.,* **183:** 2605-2613.

Yoshida, A., Takayaaki, Y. and Hishimure, T. (1996) Enzyme immunoassay for phycocyanin as the main component of *spirulina* color in foods. *Biosci. Biotechnol. and Biochem.,* **60** (1): 57-60.

Yoshida, N., Ikeda, R. and Okuno, T. (2005) Identification and Characterization of heavy metal resistant unicellular algae isolated from soil and its potential for phytoremediation. *Environ. Pollut.,* **7:** 22-27.

Zaccaro de Mule, M.C., Carie, G., Cano, M., Plama, M. and Colombo, K. (1999) Effects of cyanobacterial inoculation and fertilizers on rice seedlings and post harvest soil structure. *Communities' cation in Soil Science and plant analysis.,* **30**(2)**:** 97-107.

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ISOLATION OF A COPPER RESISTANT STRAIN OF A CYNOBACTERIUM, *NOSTOC CALCICOLA* BREB

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ABSTRACT

A tolerant strain of the diazotrophic cynobacterium, *Nostoc calcicola* was isolated in the laboratory which is capable to grow at 70µm [illegible]. A further study on characterization of this mutant is under process.

Table : 00 Figure : 00 References : 06

KEY WORDS: Bioremediation, Copper, *Nostoc calcicola*, Resistant.

Introduction

Cynobacteria are Gram -ve prokaryotes that perform oxygenic photosynthesis like plants. These cells are important in increasing nitrogen fertility in paddy fields. In our investigation we isolated a copper resistant strain that may be used in bioleaching programmes.

Materials and methods

Nostoc calcicola, a local isolate of rice field, from Algal Research Laboratory, Centre of Advanced study in Botany, Banaras Hindu University, Varanasi (U.P.)-INDIA, was cultured in 250 ml Erlen-mayer flask containing 100 ml Allen-Arnon's combined nitrogen free growth medium. The cultures were kept in culture room, which was illuminated by two 40W fluorescent tubes for 16 hrs daily. The culture received 2500-3000 lux light intensity and a temperature of 26+-1°C.

Isolation of Copper-resistant strain

Log phase population of present algae *Nostoc calcicola* with a cell density of approximately $1x10^5$ cells per ml was seeded on nutrient agar prepared in nitrogen free medium containing different copper levels (0-100µm) and subsequently shifted to growth conditions in culture room[1]. As cynobacterial cells could not grow in 40µm Cu, this formed the basis of isolation of colonies, growing in the presence of 70µm Cu under diazotrophic condition. There was general methodology for scoring the copper resistant strain[4]. The Cu^r strain clones grown as nutrient plates arose with a frequency of $2x10^{-7}$ as calculated[5] and all of these were adopted for further studies. Its stability was checked through replating on nutrient plates containing 70µm Cu. This population was grown in bulk.

Observation

A copper resistant strain of *Nostoc colcicola* was obtained that was able to grow at 70µm copper whereas 5µm Cu^{2+} was found to lethal for growth in *Nostoc calcicola* cells.

Discussion

The acquisition of heavy metal resistant in algae was known previously[6]. In some case heavy metal efflux was the major mechanism of heavy metal tolerance[2] further characterization of this strain would clearly result about the reason of Cu-tolerance in present organism.

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References

1. ALLEN, M.B. AND ARNON, D.I. (1955) Studies on nitrogen fixing blue green algae. Growth and nitrogen fixation by *Anabaena cylindrica. Plant Physiol.*,**30**: 366-372.
2. PANDEY, P.K., SINGH, C.B. AND SINGH, S.P. (1992) Cu uptake in a cynobacterium: Fate of selected photochemical reactions. *Curr. Microbiol.,* **24**:35-39
3. SINGH S.P., VERMA,S.K., SINGH, R.K. AND PANDEY, P.K. (1989) Copper uptake by a free and immobilized cynobactrium. *FEMS Microbiol. Lett.*,**60**:193-193.
4. SINGH H.N., AND SINGH, H.N. (1978) An Azide-resistant mutant of the blue green algae, *Nostoc muscorum* producing heterocysts and nitrogenase in the presence of fixed inorganic nitrogen source. *Arch. Microbiol.* **14**: 1341-1346.
5. STEWART, W.D.P. AND SINGH, H.N. (1975) Transfer of Nitrogen fixing nif gene in blue green algae *Nostoc muscorum*. *Biochem. Biophys. Res. Common.* **62**: 62-69.
6. YNEEVA Z, ZOS, Z. AND GAZAE J. (1982) Reduced cadmium transport determined by a resistant plasmid in Staphylococcus aureus. *J. Bacteriol*; **147**: 304-314